The Acute, Subchronic and Reproductive Toxicity of Guan-mu-tong (Caulis Aristolochiae Manshuriensis) and Ma-dou-ling (Fructus Aristolochiae)

by

Chan Po Wai

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Thesis Committee:

Prof. Paul P.H. But (Supervisor)

Prof. Vincent E.C. Ooi (Internal Examiner)

Prof. W. Ge (Internal Examiner)

Prof. Albert W.N. Leung (External Examiner)

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Abbreviations

AA	aristolochic acid
AA-I	aristolochic acid I
AA-II	aristolochic acid II
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BEN	Balkan endemic nephropathy
CHN	Chinese herbs nephropathy
i.g.	intragastric
i.v.	intravenous
PD_1	day-1 of pregnancy
PD_{10}	day-10 of pregnancy
PD_{16}	day-16 of pregnancy
SF. U/ml	Sigma-Frankel Units/ miniliter

Abstract

Members of the genus of *Aristolochia* have been used as medicines both in the Orient and the West. However, nephrotoxic response to several *Aristolochia* species was revealed in recent decades. Aristolochic acid, a known nephrotoxic and carcinogenic agent, was suspected to be the bioactive compound responsible for the toxic effects. Two renal diseases, the Balkan endemic nephropathy (BEN) and Chinese herbs nephropathy (CHN), have been traced to *Aristolochia* species. This project was designed to investigate the toxicity of two Chinese herbs: Guan-mu-tong 關木通 (Caulis Aristolochiae Manshuriensis) and Ma-dou-ling 馬兜鈴 (Fructus Aristolochiae). These two herbs are derived from the stem of *Aristolochia manshuriensis* and the fruit of *A. debilis* or *A. contorta*, respectively.

In the present study, aqueous extracts of Guan-mu-tong and Ma-dou-ling were used in the acute, subchronic and reproductive toxicity tests. The results from the toxicity tests showed that Ma-dou-ling had a higher toxicity than Guan-mu-tong. In acute toxicity test, no lethal dose could be determined for Guan-mu-tong as even the highest possible dosage (337.72 g/kg i.g.) did not cause 100 % mortality in mice, whereas LD_{50} of Ma-dou-ling with 95 % confidence limit was found to be 64.74±1.22 g/kg i.g. The cause of death in acute toxicity of Ma-dou-ling was probably acute renal failure. For the 3-month subchronic toxicity test, Guan-mu-tong did not exert any toxic effects on female rats while Ma-dou-ling caused damages to the kidney, liver and forestomach. Urinalysis revealed an elevated urine volume, urinary protein contents and glucose contents in the

treatment groups of Ma-dou-ling. A raised serum alanine aminotransferase activity was found in the 10 g/kg i.g. group of Ma-dou-ling after 1.5- and 3-month subchronic test. Papillomas and carcinomas with metastasis were found in the 4 and 10 g/kg i.g. groups after the 3-month subchronic test of Ma-dou-ling. Reproductive toxicity tests revealed that Guan-mu-tong had a mild effect on the fertility rate in mice while Ma-dou-ling exerted at a relative lower dosage, a 100 % anti-fertility effect at 19.2 g/kg i.g. Quantitative analysis with high performance liquid chromatography showed a higher content of aristolochic acid in the aqueous extract of Ma-dou-ling (about 459 μg/g of crude drug) than that in Guan-mu-tong (about 66 μg/g of crude drug). The lower aristolochic acid content in the latter may account for the relatively lower toxicity in the three toxicity tests.

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Chapter One: Introduction

1.1 Objective and scope of the project

Natural herbal medicines have long been used as traditional remedies and food supplements for health care in both the Orient and the West. At present, they are applied to treat diseases in the empirical ethnomedical therapies and modern medical treatments. During the last decade, there is growing interest in the applications of natural herbal medicines due to the continual discovery of bioactive components from natural herbs. Increasing numbers of medical reports have documented cases of diseases that could not be cured by modern Western medication were relieved by herbal medicine with satisfactory results (Sheehan and Atherton, 1992; Sheehan et al., 1992). Yet there are also numerous case reports about adverse reactions to botanicals (But et al., 1994, 1996; Cosyns et al., 1991, 1994; De Smet, 1992; Ivić, 1970; Kane et al., 1995; Tai et al., 1992; Vanherweghem et al., 1993).

One of the major causes of adverse reactions to botanicals is the erroneous substitution or accidental contamination of toxic botanicals (But, 1993, 1994; But and Kan, 1995; De Smet, 1992; Ng et al., 1991). Balkan endemic nephropathy (BEN) in Balkan region reported by Ivić (1970) and Chinese herbs nephropathy (CHN) in Belgium reported by Vanherweghem et al. (1993) serve to illustrate the possible dangers of these causes. In the Balkan episode, Ivić (1970) reported the peculiar prevalence of an endemic interstitial nephropathy, also known as the Balkan endemic nephropathy (BEN) in the

Balkan Peninsula and the bordering part of Yugoslavia, Romania and Bulgaria. The incriminated causal agent was *Aristolochia clematitis* which contains a potent nephrotoxin, aristolochic acid. It was postulated that the seeds of *A. clematitis,* a weed in wheat fields, contaminated the flour obtained from wheat which was consumed by peasants of the epidemic region (Ivić, 1970). In the Belgian case, problems arose form the fact that roots of *Stephania tetrandra* S. Moore (Menispermaceae) were erroneously substituted by another more potent herb in the preparation of a slimming regimen. Roots of *Aristolochia fangchi* Y.C. Wu ex Chow et Hwang (Aristolochiaceae), which contains aristolochic acid, was incriminated as the etiologic agent in CHN (But, 1993; Cosyns et al., 1994; Vanhaelen et al., 1994; Vanherweghem et al., 1993).

Some researchers have correlated BEN and CHN in clinical, morphological, pathological and histoanatomical aspects (Cosyns, 1994; Depierreux, et al., 1994; Kabanda et al., 1995). Their peculiar similarity in these aspects has drawn considerable attention from medical professions and resulted in the incrimination of the common etiologic plant species, *Aristolochia* (Cosyns et al., 1994; Kabanda et al., 1995). This project was designed to investigate another two Chinese herbs derived from *Aristolochia* species, Guan-mu-tong (Caulis Aristolochiae Manshuriensis) and Ma-dou-ling (Fructus Aristolochiae), in order to evaluate the toxicological profile of the Chinese herbs of *Aristolochia* species. Chemical analyses of the two herbal medicines were performed to evaluate the aristolochic acid contents. Different aspects of toxicity of the herbs were then investigated, including acute, subchronic and reproductive toxicity. Histological study was also performed in both acute and subchronic toxicity studies to investigate the

pathogenesis of the two herbal medicines and compare them with those of BEN and CHN.

1.2 Literature review

1.2.1 Balkan endemic nephropathy (BEN)

Balkan endemic nephropathy (BEN), a chronic tubulointerstitial disease, was described as unique because of its high prevalence in geographical distribution, clinical course and histopathology. The epidemic area is located along tributaries of the River Danube, and is highly restricted along the bordering parts of Yugoslavia, Rumania and Bulgaria. Since 1956, BEN has been documented in numerous literature (Hall, 1992; Hall et al., 1965; Hall and Dammin, 1989; Petrinska-Venkovska, 1965; Polenaković and Stefanović, 1991; Stefanović and Polenaković, 1991; Tanchev et al., 1956). These literature revealed that all patients from the epidemic area had a common and peculiar pattern of clinical syndromes and renal patho-functional changes. The initial clinical state was normal for a certain long period of time except an earlier manifestation of tubular proteinuria. The patient became sick only in the late stage of renal failure because of the signs of anaemia. Other clinical features included impaired concentration capability of kidney and absence of leukuria and hematuria. In advanced stage, it led to progressive renal failure without salt retention or hypertension. The patients finally died of uremia. Autopsy and biopsy study revealed that all patients had a unique tubulo-interstitial lesion and fibrosis without significant involvement of the glomeruli or the vasculature. They all had a symmetrical reduction in kidney size. Another feature of BEN was its high

frequency association with papillary transitional cell carcinoma of the renal pelvis and ureter.

Different etiologic agents were suspected to be the cause of BEN, including lead, cadmium, silica, bacteria, viruses, microtoxins, plant toxins, genetic factors and immune mechanisms (Hall, 1992; Hall and Dammin, 1989; Polenaković and Stefanović, 1991; Radonic and Radosevic, 1992; Stefanović and Polenaković, 1991). One plausible candidate is *Aristolochia clematitis* L. Ivić (1970) conducted a series of field investigation in the epidemic area. He observed that seeds of a weed, *A. clematitis*, were reaped and threshed together with wheat by the peasants. The flour produced was therefore contaminated with seed-meal of *Aristolochia clematitis* which contained the potent nephrotoxic agent, aristolochic acid. He further carried out an experimental investigation using rabbits fed with the contaminated flour. The pathoanatomical and pathohistological examinations revealed similar renal changes characteristic of BEN. Nevertheless, another study done on patients of BEN showed that there was no sufficient demonstration of the presence of aristolochic acid in the blood, urine and kidney tissue of the patients (Hall and Dammin, 1978).

1.2.2 Chinese herbs nephropathy (CHN)

Cases of rapidly progressive interstitial renal fibrosis has been reported in 48 women who have followed a slimming regimen containing Chinese herbs in the same clinic in Belgium. The clinic involved has specialized in slimming regimens for more

than 15 years. During this period, it applied a formula in the slimming regimen for clients without any side-effects reported. However, the formula was changed in 1990. Belladonna extract, Han-fang-ji (Radix Stephaniae Tetrandrae, root of *Stephania tetrandra*) powder and Hou-po (Cortex Magnolia Officinalis, bark of *Magnolia officinalis*) powder were added to the slimming preparation. The renal problem was found to occur after changing the formula. In early 1992, two young women who have followed the new formula of the slimming regimen were admitted to hospital due to renal failure and were started on dialysis immediately. Soon later, another seven women following the same slimming regimen were identified to have renal failure (Vanherweghem et al., 1993). Up till 1994, 71 cases of the progressive interstitial renal fibrosis have been identified and 35 of them were on renal replacement therapy (Vanhaelen et al., 1994; Vanherweghem, 1994). The clinical findings of the patients included tubular proteinuria, early and severe anemia, normal blood pressure and aseptic leukocyturia. Autopsy and biopsy study on the patients revealed that they had similar morphological and pathological patterns: reduction in kidney size, extensive cortical tubular atrophy, loss of tubules in the outer cortex, and interstitial fibrosis without glomerular lesions. Their renal function deteriorated rapidly at the end stage, leading to terminal renal failure. Papillary transitional cell carcinomas in pelviureteric urothelium was also observed (Cosyns et al., 1994 a, 1994 b; Vanherweghem, 1994; Vanherweghem et al., 1993).

Chemical analysis of the capsules of the slimming regimen was performed as patients reported to have renal failure. Thin layer chromatographic studies confirmed the presence of Hou-po in both the medication and the powder offered by the supplier.

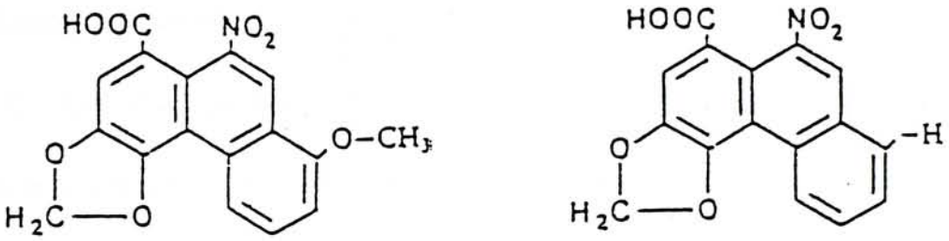
However, tetrandrine, which is the characteristic alkaloid of *S. tetrandra,* could not be detected in either the medications or the powder supplied by the Belgian import company. Instead, a third unidentified alkaloid-liked chemical which was claimed by Vanherweghen et al. (1993) not to be aristolochic acid was detected. At the meantime, analysis of the so-called tetrandra powder and the new medications performed in the Chinese Medicinal Material Research Center (CMMRC) at The Chinese University of Hong Kong showed the presence of aristolochic acid and proved the herb to be *A. fangchi*. It was proposed that Han-fang-ji 漢防己 (of *S. tetrandra*) was erroneously substituted with Guang-fang-chi 廣防己 (of *A. fangchi*) which contains the potent nephrotoxic agent, aristolochic acid (But, 1993; Vanhaelen et al., 1994).

Similar in clinical and histological findings to CHN, another case of rapidly progressive interstitial renal fibrosis was reported by Peña et al. (1996). A man was discovered to have an end stage renal failure on admission to hospital after a chronic intake of a decoction of *A. pistolochia* for four years. Clinical and histopathological findings all showed the marked characteristic of CHN.

1.2.3 Aristolochic acid.

Aristolochic acid (AA) is a yellow, bitter and acidic substance which can be found in most *Aristolochia* species. It refers to a mixture of several related nitrophenanthrene derivatives, which include aristolochic acid I (AA-I) and aristolochic acid II (AA-II) as the two principle constituents (Figure 1.1). Aristolochic acid I is 3,4-methylenedioxy-8-

methoxy-10-nitrophenanthrene-1-carboxylic ($C_{17}H_{11}NO_7$, M.W. 341.276), whereas aristolochic acid II differs in lacking the methoxy group ($C_{16}H_9NO_6$, M.W. 311.250). Other minor components are aristolochic acid III, aristolochic acid IIIa, aristolochic acid IV and aristolochic acid IVa (De Smet, 1992).



AA-I AA-II

Figure 1.1: Structure of the aristolochic acid-I (AA-I) and aristolochic acid-II (AA-II)

AA has attracted much attention since its pharmacological action was first discovered. It has once been identified to have antineoplastic activity, thus leading to extensive study of this substance (Filitis and Massagetov, 1961; Kupchan and Doskotch, 1962). It was also reported to show anti-inflammatory action. Its anti-inflammation properties had been utilized by pharmaceutical company to prepare medications in Germany (Abel and Schimmer, 1983).

However, as the discovery of its mutagenic (Robisch et al., 1982) and carcinogenic properties (Mengs et al., 1982) was reported, all the pharmaceutical preparations containing AA were withdrawn and the toxic potency of AA started to be

extensively studied. It was found to have direct mutagenic action in *Salmonella typhimurium* strains in the Ames test (Gotzl and Schimmer, 1993; Pezzuto et al., 1988; Robisch et al., 1982; Schmeiser et al., 1984) and in *Drosophila* tests (Frei et al., 1983). Another finding obtained by Abel and Schimmer (1983) showed that AA increased structural chromosome aberrations as well as sister chromatid exchanges in *in vitro* cytogenetic test on human lymphocytes. Its *in vivo* genotoxic activity in mammals was demonstrated by Mengs and Klein (1988) who found that AA was able to increase the micronucleated polychromatic erythrocytes in mice. In addition, Pezzuto et al. (1988) revealed the cytostatic potential of AA using cultured *Salmonella* species cells. Other studies on AA have proven its carcinogenic effects in rodents. Subchronic or chronic treatment of AA in rodents showed that it could induce neoplasms in the forestomach and other sites depending upon dose and time. Hyperplasia, papillomas and invasive squamous cell carcinomas in forestomach with formation of metastases were observed in rats at dosages as low as 0.1 mg/kg i.g. of AA. Hyperplasia, papillomas or carcinomas were also found in the transitional epithelium of the renal pelvis and the urinary bladder while anaplasia were noted in tubular epithelium of renal cortex (Mengs, 1983, 1988; Mengs et al., 1982, 1992). Further research on the tumor formation showed that DNA adducts formed by AA was found in target and nontarget organs in rodents (Fernando et al., 1992, 1993; Pfau et al., 1990; Schmeiser et al., 1990). In addition, its nephrotoxic action was revealed in rodents, rabbits and human (Mengs, 1987; Mengs and Stotzem, 1992, 1993; Peters and Hedwall, 1963). Massive tubular necrosis were observed in either acute or subchronic toxicity tests in rodents. Proteinuria and glucosuria were reported in urinalysis of the experimental rodents (Mengs, 1987; Mengs and Stotzem, 1992, 1993).

According to Wang and Zheng (1984), AA was shown to have anti-fertility effect. It showed effective anti-implantation effect in mice at a dosage of 3.7 mg/kg i.g. but not in rats (Kong et al, 1986). On the other hand, studies conducted by Che et al. (1984) found AA was inactive in fertility-regulating activity of rats.

In more recent literature, AA was reported to have inhibitory actions on two human immunodeficiency virus reverse transcriptase (HIV-RT) and equine infectious anemic virus reverse transcriptase (EIAV-RT) (Tang et al., 1994).

1.2.4 Guan-mu-tong

1.2.4.1 Plant

Guan-mu-tong (關木通), also known as Caulis Aristolochiae Manshuriensis, refers to the dried stem of *Aristolochia manshuriensis* Komar. (Family Aristolochiaceae) (Figure 1.2). The plant is a large woody climber with grayish green bark. Leaves are alternate, heart-shaped and densely hairy on the lower surface. Flowers are tubular and borne singly at the axil on short branches. The fruit is a cylindrical capsule with 6 angular margins. Seeds are triangular and pale grayish brown in color. Flowering season is in May while fruiting season is in August to September (Chinese Herbal Medicine Editing Committee of PRC, 1975; Pharmacopoeia Committee of PRC, 1992; Nanjing Pharmacy College, 1976).

A. manshuriensis is widespread in northern and western China. It grows in shaded, humid places of valley (Nanjing Pharmacy College, 1976).

Figure 1.2: Guan-mu-tong

1.2.4.2. Traditional uses

Guan-mu-tong has a bitter taste and cold property and is noted to be slightly toxic. Its traditional uses described in literature include clearing "heat", relieving dysuria, promoting diuresis, and stimulating menstrual discharge and the flow of milk (lactagogue action). It is prescribed for the treatment of such symptoms as ulcers in the mouth and on the tongue, dark urine, edema, painful difficult urination, excessive leukorrhea, amenorrhea, lack of lactation, and acute arthritis due to "damp heat". The conventional dosage is 3 to 9 g (Chang and But, 1986; Nanjing Pharmacy College, 1976; Pharmacopoeia Committee of PRC, 1992).

1.2.4.3 Chemical constituents

The herb contains aristolochic acid I, II, ,IIIa, IV, IVa, aristoloside, deblic acid, magnoflorine, β-sitosterol, oleanolic acid, and hederagenin, etc. (De Smet, 1992; Lou et al., 1995; Yang, 1993).

1.2.4.4 Pharmacological studies

1.2.4.4.1 Diuretic action

Intragastric injection of 20 g/kg of a decoction promoted diuretic action in rats. Experiments on anesthetized rabbits also showed diuretic action by 1g/kg i.v. and the urinary excretion of K^+, Na^+ and Cl^- ions from the treatment group was significantly higher than from that of the control group (Chang and But, 1986).

1.2.4.4.2 Action on the heart and blood vessels

Cardiotonic action on the isolated toad or guinea pig heart was exhibited by Guan-mu-tong decoction. Intravenous injection of 0.5-2 g/kg of the decoction in anesthetized dogs or rabbits was reported to elevate the blood pressure which was followed by prolonged hypotensive effect (Yang, 1993).

1.2.4.4.3 Action on smooth muscles

The decoction was found to excite isolated small intestines of mice but showed inhibition on uteri in both non-pregnant and pregnant mice (Nanjing Pharmacy College, 1976).

1.2.4.5 Reported adverse cases

Overdoses of Guan-mu-tong would cause the following toxic effects: dizziness, headache, edema, oliguria, and vomiting. Clinical analysis of the patients revealed renal pathological changes and acidosis. Extreme cases may die from acute renal failure and uremia (Yang, 1993).

1.2.5 Ma-dou-ling

1.2.5.1 Plant

Ma-dou-ling (馬兜鈴), also known as Fructus Aristolochiae, refers to the dried ripe fruit of either *Aristolochia contorta* Bunge. or *A. debilis* Sieb. et Zucc. (Family Aristolochiaceae). Both plants are perennial climbing herbs with smooth, supple and slender stems; leaves are alternate, petiolate, auriculate. Flowers are irregular, the tube being globular with the base inflated but the top narrowed. Fruits of both species are 6-lobular, 3-7 cm long, 2-4 cm in diameter with numerous compressed seeds in each locule. Seeds are thin, obtuse-triangular or fan-shaped, 6-10 mm long, 8-12 mm wide, winged and pale brown in color. *A. contorta* differs from *A. debilis* in having clusters of three to ten flowers at the axils of the leaves.

Flowering season of both species is in July and August while fruiting season is from September to October (Nanjing Pharmacy College, 1976; Pharmacopoeia Committee of PRC, 1992; Xu and Xu, 1994).

A. contorta is mainly widespread in northern China, while *A. debilis* in southern China. Both species grow along paths, in damp places along stream banks and at the edge of woodlands, and in hedges (Nanjing Pharmacy College, 1976; Xu and Xu,1994).

Figure 1.3: Ma-dou-ling

1.2.5.2 Traditional uses

Ma-dou-ling has an arid and bitter taste, and is regarded to have a cold property and a little toxicity. Its folk uses include clearing "heat" from the lung, and relieving cough (antitussive) in bronchitis and pneumonia, being an expectorant in asthma, and removing "heat" from the large intestine for the treatment of bleeding, swollen and painful hemorrhoid. The conventional dosage is 3 to 9 g (Chang and But, 1986; Nanjing Pharmacy College, 1976; Pharmacopoeia Committee of PRC, 1992; Yang, 1993).

1.2.5.3 Chemical constituents

The herb contains aristolochic acid I, II, IIIa, IVa, B, 7-hydroxy-aristolochic acid I, 7-methoxy-aristolochic acid, aristolochine, magnoflorine, β-sitosterol, aristolochinic acid, and debilic acid (De Smet, 1992; Yang, 1993).

1.2.5.4 Clinical and pharmacological studies

1.2.5.4.1 Clinical action:

A compound formula containing Ma-dou-ling was effective in the treatment of 126 cases of chronic bronchitis. After 6 courses of treatment, 67 cases were satisfactorily controlled and 40 cases showed significant improvement (Yang, 1993). Another report revealed that a compound formula containing Ma-dou-ling was effective in the treatment of 94 cases of chronic bronchitis (Chang and But, 1986).

1.2.5.4.2 Pharmacological studies:

(i) Antitussive action

Coughing induced by ammonium hydroxide spraying method in mice and by electric stimulation of nerve in cats could be reduced by Ma-dou-ling at 10 g/kg i.g. and 2 g/kg i.p., respectively (Wu and Xiong, 1989; Xu and Xu, 1994).

(ii) Expectorant action

In an experiment measuring the respiratory mucus secretion of anesthetized rabbits, the decoction of Ma-dou-ling was proved to have a weak expectorant action at the

dosage of 1 g/kg i.g. (Chang and But, 1986). Another similar study on rats also demonstrated a weak expectorant action at the dosage of 4 g/kg (Xu and Xu, 1994).

(iii) Action on smooth muscles

In vitro perfusion studies showed that the constriction of isolated bronchus of guinea pig induced by acetocholine, histamine, or by pilocarpine could be antagonized by 1 % extract of Ma-dou-ling. Therefore, the herb is regarded to have anti-asthma action (Jiangsu College of New Medicine, 1956; Yang, 1993). Besides, it could cause constriction on the peripheral blood vessels, intestinal tracts and uteri of animals (Yang, 1993).

(iv) Antifungal action

Extract of Ma-dou-ling demonstrated antifungal action against Schlemm's *Dermatomyces favosa, Microsporum audouini* and *Microsprum lanosum in vitro* (Chang and But, 1986; Yang, 1993).

1.2.5.5 Reported adverse cases

There are few reports on the adverse reaction induced by Ma-dou-ling. Overdose is the main reason of the toxic reaction. The symptoms of intoxication are nausea, vomiting, abdominal pain, diarrhea, hematuria, proteinuria, dysnea, and hypotension (Yang, 1993). A man who had chronic bronchitis took a decoction of 30 g of Ma-dou-ling and then developed symptoms of nausea, dizziness, tremor, dysnea, and severe vomiting with blood. Clinical findings included dehydration and acidosis (Ying, 1983).

Besides, a woman was hospitalized due to severe vomiting with dehydration after taking a decoction of 15 g of Ma-dou-ling for the treatment of hemorrhoid (Siu, 1989).

1.3 Chemical analysis

Analytical methods were necessary to identify the chemical constituents of the crude herbal drugs qualitatively and quantitatively before the toxicological studies were conducted. In the present study, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were chosen to obtain information of the major chemical components of the two herbal drugs of interest.

1.3.1 Thin layer chromatography

Thin layer chromatography (TLC) refers to the process of separating the constituents of a mixture by permitting a solvent system to flow through a thin layer of adsorbent on which different substances are selectively separated into distinct spots or bands. The separation of the constituents of the sample mixture depends on the interaction of the adsorbent, solvent systems and the solutes in the sample mixture, which in turn rely upon the molecular structure of the three components. The individual separated spots are detected by visible light or UV visualization, by fluorescence, radioactivity or by staining the components with a reagent. A series of sample standard is usually spotted on the same plate for comparison with the sample drug in terms of relative

migration distance (Rf) value and the distinct color of the spots (Stevenson and Bridges, 1986; Wagner, 1996).

The technique of TLC is simple, rapid and efficient for the identification of the sample herbal drugs but cannot offer an accurate and rapid quantitative assessment; nevertheless it provides a preliminary information for further sophisticated analytical procedures of the herbal drugs (Stevenson and Bridges, 1986). In studying Guan-mu-tong and Ma-dou-ling, the confirmation of the presence of AA was performed by TLC before quantitative analysis.

1.3.2 High performance liquid chromatography

High performance liquid chromatography (HPLC) refers to the process of separating the constituents of a mixture by pumping the sample with a stream of solvent system into a tightly packed column under a high pressure. As in TLC, the separation of the constituents depends on the different partition rates between the mobile (the solvent system) and stationary phases (the adsorbent), which in turn depend on the nature, composition and structure of the components involved. There are different modes of HPLC, among which bonded phase chromatograph (BPC) in reverse phase (RP) separation is the most widely used. The packing material for this type used is silica bonded with alkyl chains of different lengths (C18 being the most common) to offer a non-polar surface. The polar nature of solvent system together with the non-polar surface of the stationary phase permit differential elution of compounds which are then analyzed

by a detector. Ultraviolet, fluorescence, electrochemical, refractive, and conductivity are five main types of detectors. The components of the mixture can then be analyzed qualitatively and quantitatively with standard calibration (Mcmaster, 1994; Stevenson and Bridges, 1986).

In the present study, HPLC of reverse phase separation is applied to further confirm the identification of AA in the herbal drugs and to quantify the AA contents for comparison with the toxic effect observed in the bioassays.

1.4 Toxicological studies

Toxicity refers to the harmful effects of substances on living systems. Different types of toxicants possess different specific target organ, mode of action and potency. In order to determine the primary and cumulative toxicity of the two herbs, short (acute toxicity) and intermediate (subchronic toxicity) term toxicological studies were conducted in the present study (Chan et al., 1992; Lu, 1996). In addition, a reproductive toxicity test was performed to evaluate the harmful effect of the herbs on pregnancy.

1.4.1 Acute toxicity

Acute toxicity is defined as "the adverse effects occurring within a short time of [oral] administration of a single dose of a substance or multiple doses given within 24 hours" (Organization for Economic Cooperation and Development, 1981). The

objectives of acute toxicity test are to provide indications of the probable target organ and the specific toxic effects as well as guidelines for further prolonged studies on the substances (Chan et al., 1992; Lu, 1996).

There are two types of parameters for the acute toxicity study: lethal and non-lethal. Median lethal dose (LD_{50}) is one of the lethal parameters. It is defined as "the statistically derived single dose of substance that can be expected to cause death in 50% of the animals" (Organization for Economic Cooperation and Development, 1981), and is usually used to classify and to compare toxicity among substances. Slopes of the dose-response curves, time to death, pharmacotoxic signs, and histopathological changes of the dead animals are other lethal parameters of the acute toxicity. Besides, non-lethal parameters should also be taken into consideration in evaluating the toxicity. Those animals that survive the test period are subjected to necropsies at the end of the observation period. Histopathologic examination of the tissues would be a source of understanding the acute toxic effect of the substances for the investigation of the reversibility of the toxicants (Chan et al., 1992; Lu, 1996).

1.4.2 Subchronic toxicity

Subchronic toxicity refers to "the adverse effects occurring as a result of the repeated daily dosing of a chemical to experimental animals for part (not exceeding 10%) of the life span" (Organization for Economic Cooperation and Development, 1981). The doses used in subchronic toxicity tests are usually lower than those in the acute toxicity

tests. This type of toxicity study at low dose exposure offers information on the cumulative effects and dose response relationship of the toxicants (Chan et al., 1992; Lu, 1996).

The major indices of subchronic toxicity are the non-lethal ones. In the present study, the following parameters selected were based on the class of the two herbs and the expected toxicity for short term risk assessment of the two herbal drugs.

1.4.2.1 Body weight

Body weight is a simple sensitive index of toxic effects. It is usually measured weekly.

1.4.2.2 Urinalysis

Urinalysis is usually included in toxicity studies when renal toxicity is suspected to be resulted from the toxicants. Urine volume and urinary protein and glucose were determined in this study. Normal kidneys reabsorb most of the water in glomerular filtrate. Polyuria or oliguria may indicate impaired renal functions. Almost all glucose in the glomerular filtrate are reabsorbed completely in the proximal tubule and thus an abnormally high urinary glucose content indicates proximal tubular damage (Zilva and Pannall, 1975). In normal subjects, small amounts of low molecular weight proteins are filtered into the glomerular filtrate but nearly all are reabsorbed and catabolized by proximal tubule. On the other hand, high molecular weight proteins cannot pass through the glomerular wall and should not be found in the glomerular filtrate as well as urine.

Thus, proteinuia may be either caused by the failure of proximal tubular reabsorption or increased glomerular permeability (Walmsley and White, 1983). An abnormally high urinary protein and/or glucose content may indicate proximal tubular or glomerular damage (Hodgson and Levi, 1994).

1.4.2.3 Clinical biochemical analysis

Biochemical analysis of the plasma or serum is important in evaluating target organ toxicity. It provides information about damages on the organ or system function by the toxicants. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were selected as indicators of liver cell damage (hepatocellular damage) in the present study. Transaminases are enzymes that transfer the amino group from an amino acid to a keto acid. AST and ALT catalyze the following reactions respectively:

$$\text{Aspartate} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{AST}} \text{oxaloacetate} + \text{glutamate}$$

$$\text{Alanine} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{ALT}} \text{pyruvate} + \text{glutamate}$$

These enzymes are normally present in the blood in relatively low concentrations. AST is a cytoplasmic and mitochondrial isoenzyme and is widely distributed in tissues including hepatocytes, skeletal muscle, myocardium, kidney, pancreas and red blood cells. A raised activity of serum AST may be due to myocardial disease, liver disease, skeletal muscle

disease, haemolysis, renal infarction, or hypothyroidism. ALT exists largely in the cytosol. It is specific for hepatocytes. Thus, a raised activity of ALT would be a good indicator of hepatocellular damage (Jones and Berk, 1979; Meites, 1977).

1.4.2.4 Organ weights

Gross pathologic examination and organ weights are obtained for test animals found dead or during necropsies at the end of the observation period. This provides a primary screening of lesion found in the organs which may then be subjected to further histopathology study. In the present study, organ weights of heart, liver, spleen, lung, kidney, stomach, and uterus were measured.

1.4.2.5 Histopathological change

Histological examination provides information of any pathological changes caused by the toxicants. It reveals the structural changes caused by toxicants. The histopathological findings, together with the biochemical and physiological data, can offer a more complete picture to the toxicity of the drugs.

In this study, conventional light microscopy study was performed on selected tissues. Hematoxylin and eosin were used to stain the specimens.

1.4.3 Reproductive toxicity

According to the Chinese literature, Guan-mu-tong and Ma-dou-ling should be used with caution in pregnancy or are even contraindicated in pregnancy (Pharmacopoeia committee of PRC, 1992; Yang, 1993). Thus, the present study included the investigation of effects of the herbal medicines on the female reproductive system during pregnancy. The numbers of animals pregnant, normal fetuses and corpus lutea were obtained to evaluate the toxic effects of the two herbal medicines on early implantation and the development of fetuses.

Chapter two: Materials and Methods

2.1 Materials

2.1.1 Chinese herbs

Guan-mu-tong (Caulis Aristolochiae Manshuriensis) and Ma-dou-ling (Fructus Aristolochiae) were supplied by a local Chinese herb wholesaler. The following voucher specimens were kept at the Museum of the Chinese Medicinal Material Research Center, The Chinese University of Hong Kong: 972251 (for Ma-dou-ling) and 972252 (for Guan-mu-tong).

2.1.2 Animals

Sprague-Dawley female rats (180–220 g), ICR male mice (18–22 g) and ICR pregnant mice (30–40 g) were supplied from the Animal House, The Chinese University of Hong Kong. They were housed under conditions of 18-20°C, humidity 54-56 % and a 12-hour light-dark cycle. They were supplied with standardized pellet food (Supastok Autoclavable Rodent Diet, Ridley Agriproducts, Australia) and tap water *ad libitum*.

2.1.3 Chemicals

Aristolochic acid

Standard aristolochic acid (AA) was purchased from Sigma (U.S.A.). It contains 52 % aristolochic acid I (AA-I) and 41 % aristolochic acid II (AA-II).

Phosphate buffer of pH 7.5

Phosphate buffer was prepared by mixing 420 ml 0.1 M disodium hydrogen phosphate (26.81 g $Na_2HPO_4.7H_2O$/L) (Ajax, Australia) and 80 ml 0.1 M potassium dihydrogen phosphate (13.61 g KH_2PO_4/L) (Ajax, Australia). The solution was adjusted to pH 7.5 by 1M sodium hydroxide or 1 M hydrochloric acid.

Sodium pyruvate

For the subchronic toxicity test of Guan-mu-tong, 1.5 mM of sodium pyruvate (Sigma, U.S.A.) in phosphate buffer of pH 7.5 was used for the serum aspartate and alanine aminotransferase assay.

DL-Aspartic acid

In subchronic toxicity test of Guan-mu-tong, 0.2 M of DL-aspartic acid, monosodium salt (Sigma, U.S.A.) in phosphate buffer of pH 7.5 was used for the serum aspartate and alanine aminotransferase assay.

α-Ketoglutaric acid

In subchronic toxicity test of Guan-mu-tong, 1.8 mM of α-ketoglutaric acid (Sigma, U.S.A.) in phosphate buffer of pH 7.5 was used for the serum aspartate aminotransferase assay.

DL-Alanine

In subchronic toxicity test of Guan-mu-tong, 0.2 M of DL-alanine (Sigma, U.S.A.) in phosphate buffer of pH 7.5 was used for the serum alanine aminotransferase assay.

2,4-Dinitrophenylhydrazine

In subchronic toxicity test of Guan-mu-tong, 1 mM of 2,4-dinitrophenylhydrazine (Sigma, U.S.A.) in 1 M hydrochloric acid was used for the serum aspartate and alanine aminotransferase assay.

Enzyme kits

Kits of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from Sigma (U.S.A.) for performing the enzyme assays in subchronic toxicity study of Ma-dou-ling.

Diagnostic glucose kits

Diagnostic glucose kits were purchased from Sigma (U.S.A.) for performing the assay in subchronic toxicity test of Guan-mu-tong and Ma-dou-ling.

10% Buffered formalin, neutral

10% buffered formalin was prepared by dissolving 6.5 g of di-sodium hydrogen phosphate orthophosphate (Ajax, Australia) and 4.0 g sodium di-hydrogen orthophosphate (Ajax, Australia) in 900 ml distilled water and then mixed with 100 ml commercial formalin (formaldehyde solution, Ajax, Australia)

2.2 Methods

2.2.1 Aqueous extraction of the Chinese herbs for acute, subchronic and reproductive toxicity tests

In each extraction of Guan-mu-tong, 300 g of dry plant material was soaked in 3.3 liters of distilled water for 1 hour, and then boiled for 1 hour. The extract was then filtered by a three-layer cotton cloth. The extract was lyophilized into dry powder for storage in a desiccater. A total of 31.20 g of dry powder was obtained from 300 g dry plant material of Guan-mu-tong, i.e. 10.4 %. The aqueous extract of Guan-mu-tong is hereafter referred to as Guan-mu-tong extract.

In each extraction of Ma-dou-ling, 100 g was soaked in 3 liters of distilled water for 1 hour, and then boiled for 1 hour. The extract was then filtered and lyophilized into dry powder in the same way as Guan-mu-tong. A total of 12.00 g of dry powder was obtained from 100 g dry plant material, i.e. 12.0 %. The extract of Ma-dou-ling hereafter referred to as the Ma-dou-ling extract.

2.2.2 Chemical analysis

2.2.2.1 Thin layer chromatography (TLC)

Ground powder of dry plant material or aqueous extract powder, 0.3 g, was refluxed for 30 minutes in 5 ml of absolute ethanol (analytical grade, BDH, England). The extract was filtered by Whatman No.1 filter paper to separate it from the plant residue. The reference mixture, standard aristolochic acid (AA), 1.5 mg was dissolved in 200 μl of absolute ethanol. The ethanolic extract of Chinese herbs and standard AA were then applied onto a silica gel 60 F_{254}-precoated TLC plates (Merck, Germany). A solvent system of toluene (Riedel-de Haën, Germany) - ethyl acetate (Merck, Germany) - methanol (Lab-Scan, England) - formic acid (Riedel-de Haën, Germany) (in volume ratio of 20:10:1:1) was used. The chromatogram was then detected at UV 254 nm (Xu and Xu, 1994).

2.2.2.2 High performance liquid chromatography (HPLC)

2.2.2.2.1 Sample preparation

Ground powder of dry plant material or aqueous extract powder, 0.5 g in 25 ml of absolute ethanol (analytical grade, BDH, England) was refluxed for 1 hour. The volume of filtrate was then adjusted to 25 ml with absolute ethanol. Standard AA, 2.3 mg, was dissolved in 5 ml of absolute ethanol, which was then diluted to 20 ml (final concentration: 0.115 mg/ml). Aliquot of the extract and standard AA solution was then filtered with 0.45 μm filter of the HPLC (Xu and Xu, 1994).

2.2.2.2.2 Equipment

The chromatographic system consisted of a quaternary pump (Quatpump, G1322A), an autosampler (ALS, G1313A), a diode array detector (DAD, G1315A) (Hewlett Packard series 1100, Germany). Instrument control and data evaluation were performed with a Chemstation software (Hewlett Packard, Germany).

The separation was performed using a thermostatted column compartment C18 column (250 mm x 4 mm i.d., 5 μm particle size) (Col. Comp, G1316A) (Hewlett Packard, Germany).

2.2.2.2.3 Reagents

Methanol (Lab-Scan, England) and acetic acid (Riedel-deHaën, Seelze, Germany) were of analytical grade. Water was deionized.

2.2.2.2.4 Chromatographic conditions

Analyses were performed using a mobile phase of methanol-water-acetic acid (70:29:1). The flow rate was 1 ml/min. The column was kept at room temperature. The monitoring wavelengths were UV 254 and 315 nm.

2.2.2.2.5 Peak identification

Chromatograms were assessed for peak area, retention time and UV scan. Identification of significant peaks was based on the relative retention times and UV scan with that of standard AA. Quantitative analysis of AA present in the samples were

performed by calculating the corresponding peak area of the samples with that of standard AA. Standard AA 2, 5, 10, 20 or 30 μl of 0.115 mg/ml was injected to obtain a calibration curve (Figure A1).

2.2.3 Assays for the toxicity

2.2.3.1 Acute toxicity

For Guan-mu-tong, ICR male mice of 18–22 g were randomly divided into groups of ten as a preliminary test served to screen the dose range. Each mouse was given a single dose of 337.72g/kg i.g. (the maximum possible dosage that can be suspended by the vehicle) in a volume of 0.2 ml/10 g body weight after 4-hour fasting. The mice in the control group received the vehicle (distilled water) in an equivalent volume.

For Ma-dou-ling, ICR male mice of 18-22 g were randomly divided into groups of ten. Each mouse was given a single dose by gavage in a volume of 0.2 ml/10 g body weight after a 4-hour fasting. The dose ranges tested were 51.2, 64, 80 and 100 g/kg i.g. The mice in the control group received the vehicle (distilled water). Due to the occurrence of advanced autolysis and cannibalism, no readable tissue sections could be obtained. An additional batch was used to obtain histopathological information of moribund animals. They were then kept under observation for general symptoms of toxicity and mortality for 21 days thereafter (Mengs, 1987).

Body weight of mice for the acute toxicity test of Guan-mu-tong and Ma-dou-ling was measured 5 times per week. Autopsies were carried out after 21 days. Kidneys were

fixed in 10% buffered formalin for histological processing. Dehydration in grading scale of ethanol, xylene and embedding in paraffin were performed according to the standard procedures. Five micron sections were cut and stained by hematoxylin and eosin (H&E), followed by mounting with balsam. Microscopic studies were then followed. Detailed procedure of the histological processing was presented in Appendix F.

2.2.3.2 Subchronic toxicity

For subchronic toxicity test of Guan-mu-tong, female rats of 180-220 g were randomly assigned to groups of ten after a 14-day acclimatization period. Each group was given daily doses of 1, 3 or 9 g/kg/day in a volume of 8 ml/kg by gavage for 3 months. Another group received the solvent (distilled water) and served as control.

For subchronic toxicity test of Ma-dou-ling, female rats of 180–220 g were randomly assigned to groups of twenty after a 14-day acclimatization period. Each group was given daily doses of 2, 4 or 10 g/kg/day in a volume of 8 ml/kg by gavage. In each group, one batch of 10 rats was administrated for 1.5 months while another batch of 10 rats was dosed for 3 months. Another group of twenty rats received the vehicle (distilled water) and served as control.

During the treatment period for either Guan-mu-tong or Ma-dou-ling, the animals were observed for external general symptoms of toxicity and mortality. Body weight was measured weekly. Urine samples were collected once a week by placing the rats in metabolic cages without food for 24 hours. Urinalysis comprised measurement of urinary

volume, color, protein (Lowry method) and glucose (enzymatic test, Sigma glucose diagnostics kit). Procedures for the protein and glucose assays were summarized in Appendixes A and B, respectively.

After 3-month treatment for Guan-mu-tong treatment and 1.5- and 3-month for Ma-dou-ling treatment, rats were anesthetized and blood samples were collected through the dorsal aorta. Serum were isolated from the blood samples for serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme assays by centrifuge at 3000 rpm for 10 minutes. Gross pathological examination were carried out and organ weights were measured. Organs were fixed in 10% buffered formalin for histological processing and microscopic studies. Procedures for the enzyme assays and histological processing were referred to in Appendixes C-F.

2.2.3.3 Reproductive toxicity

ICR pregnant mice of 30–40 g were supplied by the Animal House at The Chinese University of Hong Kong in the first morning when the presence of post-coital plug was observed. These mice were considered to be on pregnancy day 1 (PD_1). They were randomly assigned to control and experimental groups, in groups of ten. The experimental groups were administered intragastrically with Guan-mu-tong or Ma-dou-ling extract by gavage according to their body weight at around 10:00 am. They were dosed daily from PD_1 to PD_{10}. The corresponding control group was given the vehicle (distilled water). The mice were autopsied on PD_{16} and the number of pregnant animals, implantation sites, normal fetuses and corpora lutea were recorded (Kong *et al.*, 1989).

2.2.4 Statistical analysis

All results are expressed as arithmetical means ± standard errors measurement. LD_{50} values were calculated by means of probit analysis. Analysis of statistically significant differences in group comparisons was first performed by means of analysis of variance or Kruskal-Wallis analysis of variance. Comparison of the treatment groups with the control group was then done by Dunnett's test (Gad and Weil, 1988).

Chapter Three: Results

3.1 Chemical analysis

3.1.1 Thin Layer Chromatography

TLC analysis of the crude drug and the aqueous extract of Guan-mu-tong and Ma-dou-ling confirmed the presence of AA as compared with the reference standard mixture. Two bands were observed as yellowish color under visible light and as dark green fluorescence under UV 254 nm, at Rf 0.40 and 0.56 in all samples as well as in the reference standard mixture (Figure 3.1 and 3.2). Other bands of different colors and at different Rf values in the herbal medicines were also observed. These bands were not identified in the present analysis.

3.1.2 High Performance Liquid Chromatography

Two peaks were found at relative retention times of 6.343 & 7.788 minutes in HPLC chromatogram of standard AA detected at UV 254 nm. The first and second peaks were identified as AA-I and AA-II respectively because of the relative amounts of these two compounds claimed by the supplier (AA-I: 52%; AA-II: 41%). Peak height and area of the first peak were higher and larger than those of the second one, implying greater amounts at the first peak (Figure 3.3). UV spectra of the two peaks exhibited maximum absorbance at about 254 and 315 nm (Figure 3.5). UV 254 nm was selected to perform the qualitative and quantitative analysis of AA in the two herbal medicines.

Two peaks matching with relative retention times and UV scan at 254 nm as those of reference compound were found in crude drug of Guan-mu-tong and Ma-dou-ling. This confirmed the presence of AA-I and AA-II in the two herbal medicines (Figure 3.3 and 3.5). AA content in the herbal medicines determined from the calibration curve (Figure A1) were tabulated in Table 3.1. The crude drug Guan-mu-tong was found to have a higher AA content than Ma-dou-ling.

Aqueous extract of Ma-dou-ling showed two peaks with the same relative retention times and UV spectra at 254 nm as that of AA-I & AA-II, while aqueous extract of Guan-mu-tong showed one peak with similar relative retention time (at about 7.8 minutes) and same UV spectrum at 254 nm as that of AA-II in the reference compound (Figure 3.4 and 3.5). The amount of AA-I was not well measured by the equipment for the aqueous extract of Guan-mu-tong because if present it was partially masked by other components in the latter extract. The AA II content was about 5-6 times higher in aqueous extract of Ma-dou-ling than in the aqueous extract of Guan-mu-tong (Table 3.2). The percentage yield of AA extracted from crude drug was much higher in Ma-dou-ling than in Guan-mu-tong (Table 3.3).

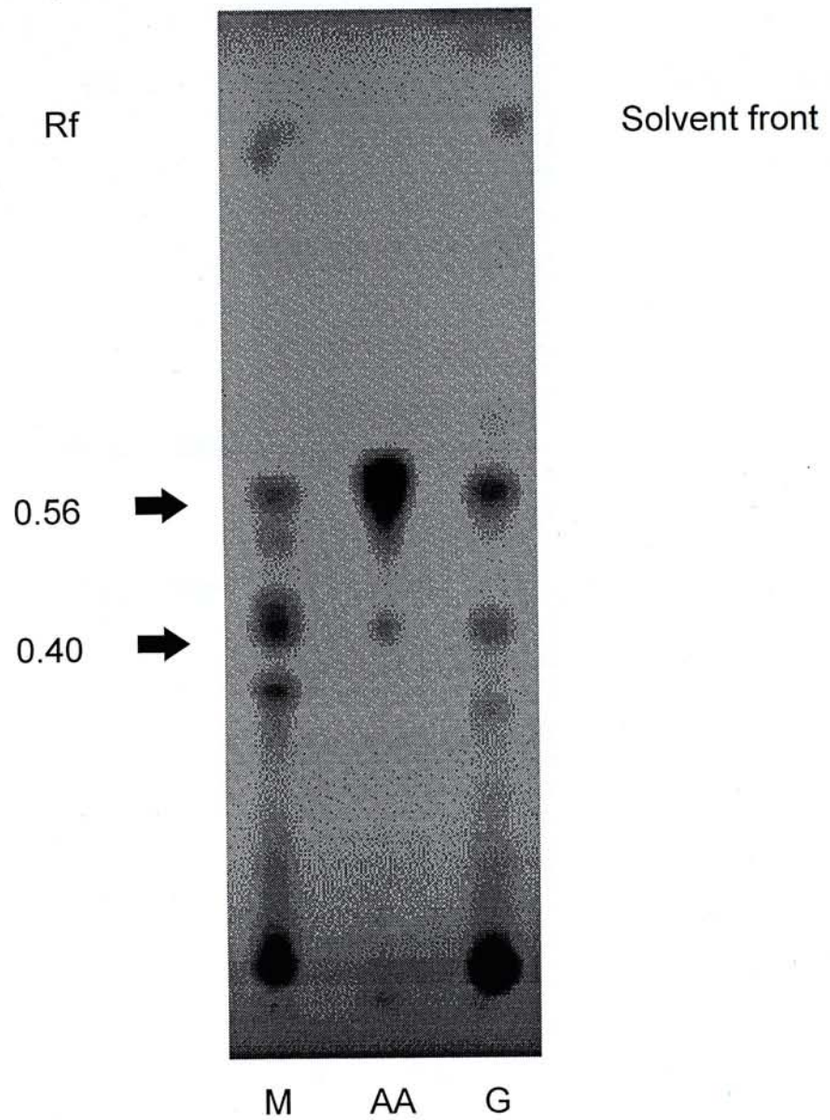


Figure 3.1: The thin layer chromatograph of standard aristolochic acid (AA) and ethanolic extract of crude drug Guan-mu-tong (G) and Ma-dou-ling (M) at UV 254 nm.

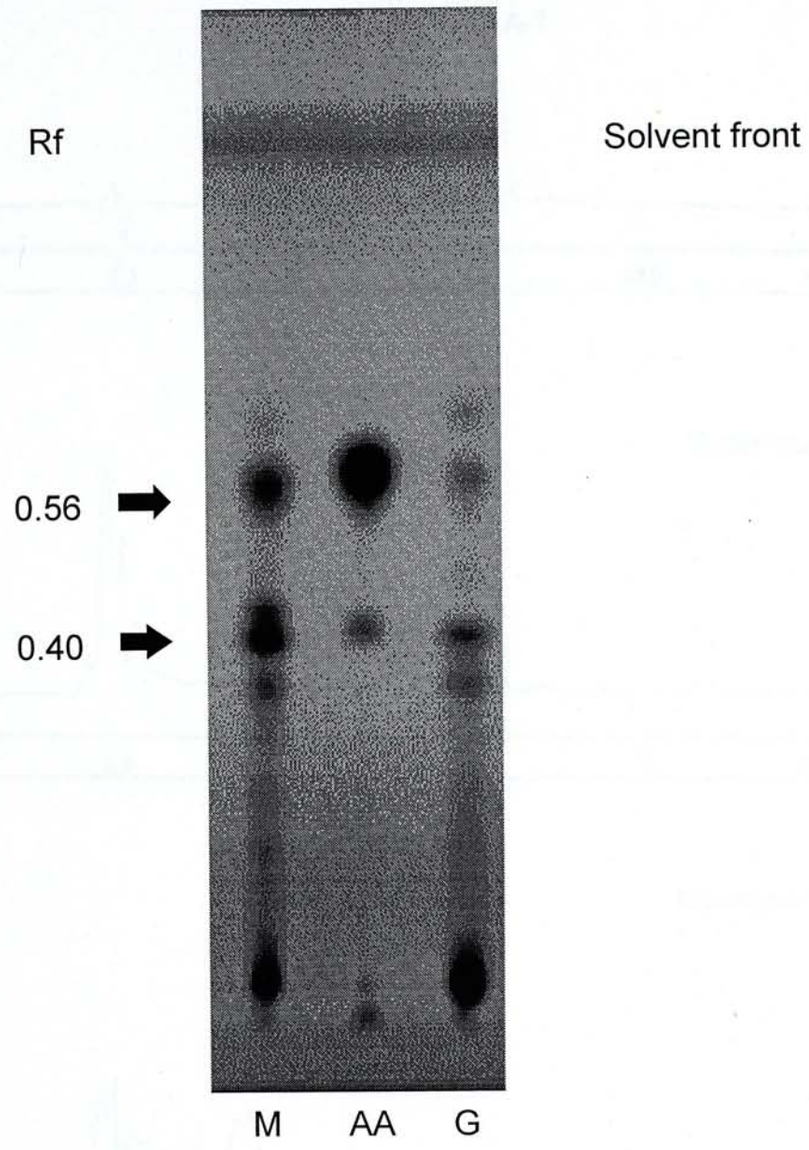


Figure 3.2: The thin layer chromatograph of standard aristolochic acid (AA) and aqueous extracts of Guan-mu-tong (G) and Ma-dou-ling (M) at UV 254 nm.

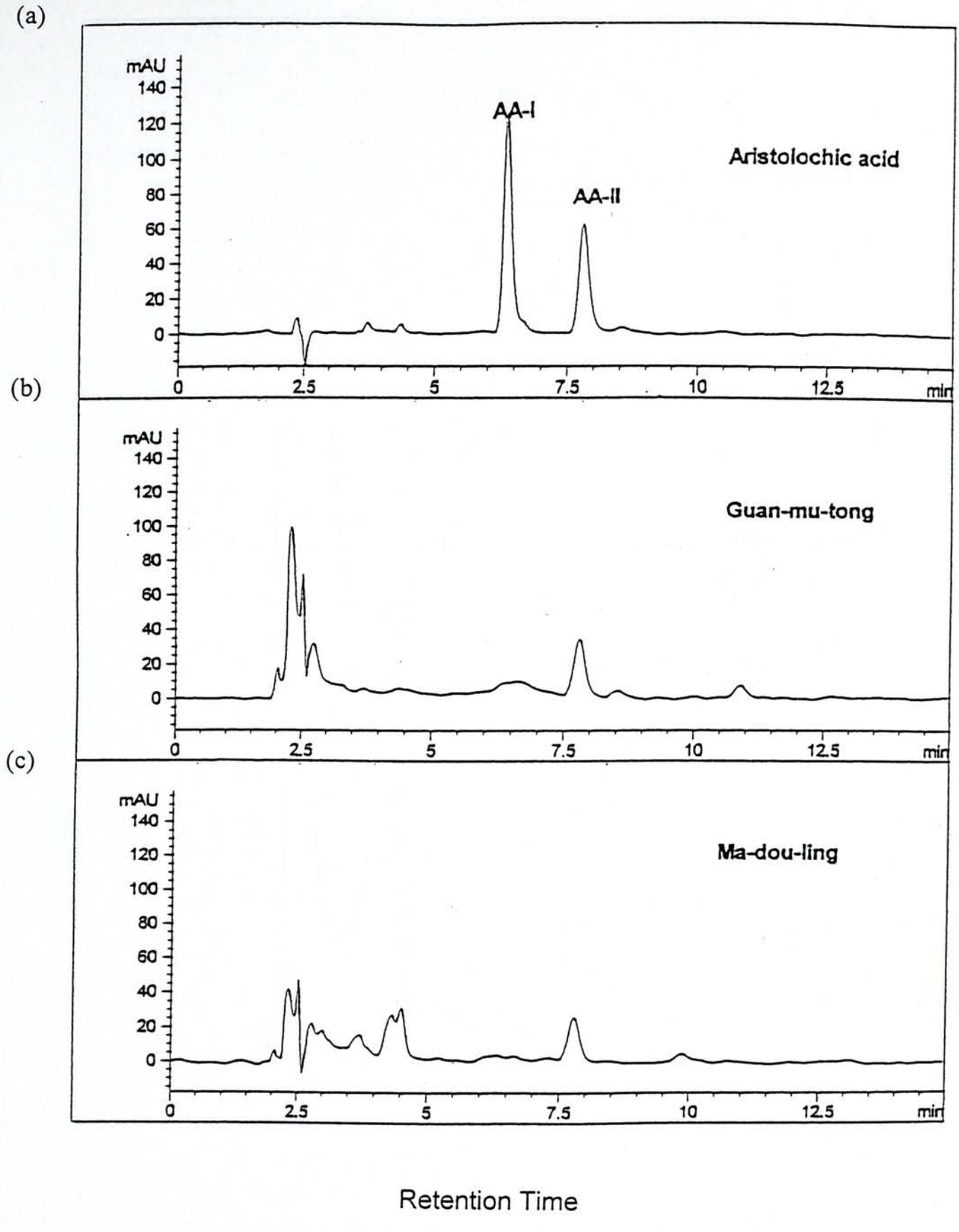


Figure 3.3: HPLC chromatogram of (a) aristolochic acid; (b) ethanolic extract of crude drug Guan-mu-tong; (c) ethanolic extract of crude drug Ma-dou-ling.

AA-I : aristolochic acid I
AA-II: aristolochic acid II

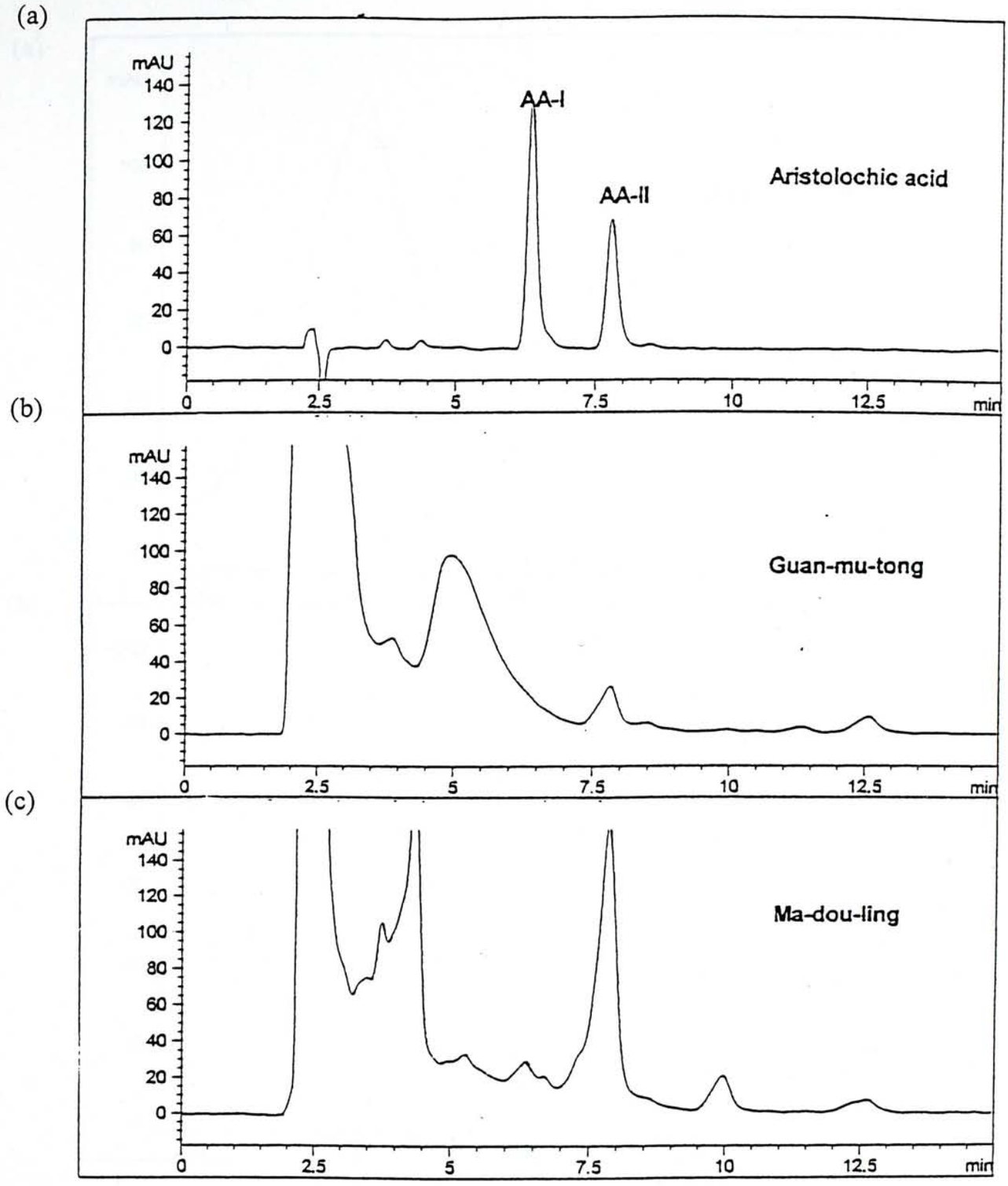


Figure 3.4: HPLC chromatogram of (a) aristolochic acid; (b) aqueous extract of Guan-mu-tong; (c) aqueous extract of Ma-dou-ling.

AA-I : aristolochic acid I
AA-II: aristolochic acid II

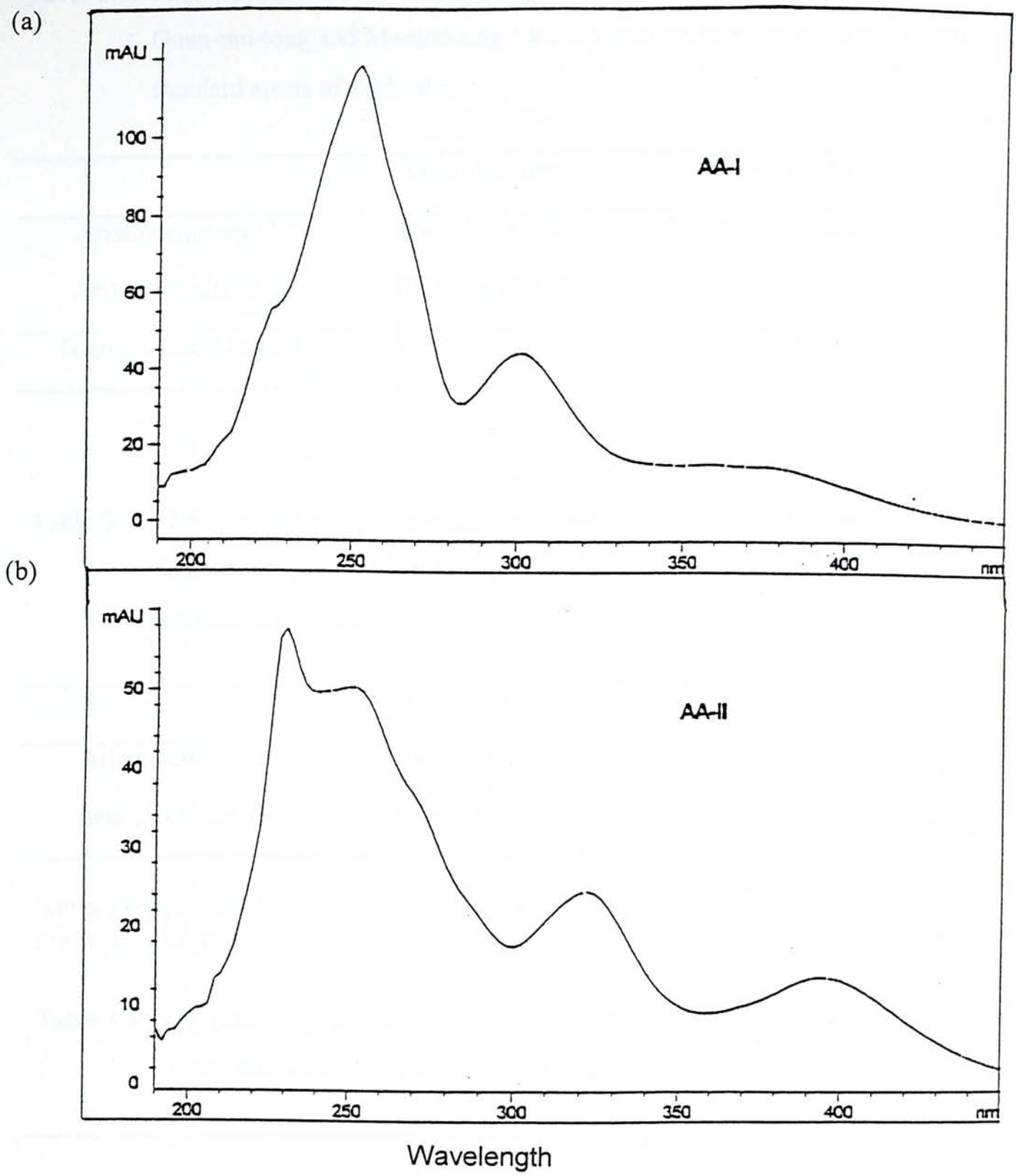


Figure 3.5: UV spectra of (a) aristolochic acid I (AA-I) and (b) aristolochic acid II (AA-II).

Table 3.1: Aristolochic acid content (μg per gram of crude drug) in ground crude drug of Guan-mu-tong and Ma-dou-ling. Values are expressed as arithmetic means ± standard errors of triplicates.

	Guan-mu-tong	Ma-dou-ling
Aristolochic acid I	296.96±13.1207	97.58±1.558
Aristolochic acid II	1055.96±14.1834	841.37±8.817
Total aristolochic acid	1352.93±20.9725	938.95±8.2013

Table 3.2: Aristolochic acid content (μg per gram of crude drug) in aqueous extract of Guan-mu-tong and Ma-dou-ling. Values are expressed as arithmetic means ± standard errors of triplicates.

	Guan-mu-tong	Ma-dou-ling
Aristolochic acid I	minute amount[a]	15.485±0.606
Aristolochic acid II	66.043±0.6890	444.130±1.205

[a] cannot be quantified due to interference by other components in HPLC but estimated to be lower than 15 μg/g.

Table 3.3: The percentage of aristolochic acid extracted into the aqueous extract from the crude drugs Guan-mu-tong and Ma-dou-ling.

	Guan-mu-tong	Ma-dou-ling
Aristolochic acid I	–[a]	15.87
Aristolochic acid II	6.25	52.79

[a] cannot be calculated due to interference by other components in HPLC.

3.2 Toxicity of Guan-mu-tong

3.2.1 Acute toxicity

After a single dose of 337.72 g/kg i.g. of Guan-mu-tong, all the ten mice were sedated. Four mice died within the first six days while the rest of the mice did not have any toxic signs and abnormal behavior except sedation. The body weight of the treatment group was affected. The control group had a larger gain in body weight (12.50±0.506 g) after 21-day observation while the survivors of the treatment group had a relatively smaller gain in body weight (3.25±1.03 g for 337.72 g/kg i.g.) (Table 3.4).

The survivors were sacrificed for autopsies on the twenty-first day after dosing. No other lesions was found in the surviving animals.

Table 3.4: Change in body weight of surviving mice on 21st day after intragastric administration a single dose of Guan-mu-tong extract.

Group (n=10)	Dosage (g/kg i.g.)	No. of survivors per no. of mice dosed	Body weight gain in gram (arithmetic means ± standard errors)
Control	-	10 / 10	12.50±0.506
Guan-mu-tong extract	337.72	6 / 10	3.25±1.03***

*** Significantly different when compared with the control group, $p < 0.001$.

3.2.2 Subchronic toxicity

During the 3-month subchronic test period, the clinical states of the rats of all the treatment groups were normal when compared with that of the control group.

3.2.2.1 Body weight

The body weight of all groups increased with time. During the 3-month subchronic test period, the body weight of all the treatment groups was not significantly different from that of the control group (Figure 3.6). In comparison with the control group, there was no significant reduction in body weight gain in the treatment groups at the end of the 3-month subchronic test period (Table 3.5).

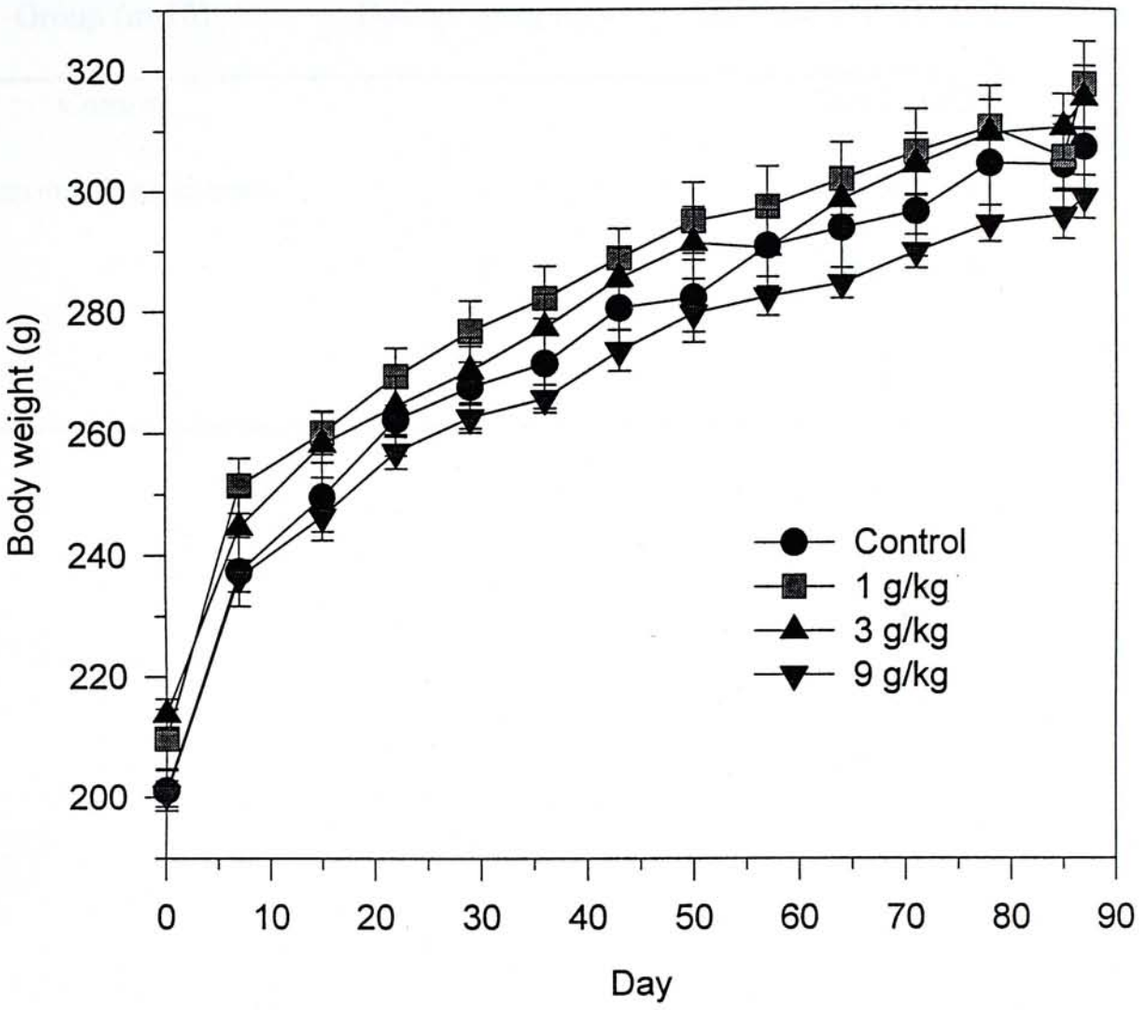


Figure 3.6: Effect of subchronic treatment of Guan-mu-tong extract on the body weight of rats.

Values are expressed as arithmetic means ± standard errors.

Table 3.5: Increase in body weight of rats after 3-month subchronic test fed with Guan-mu-tong extract. Values are expressed as arithmetic means ± standard errors.

Group (n=10)	Dosage (g/kg i.g.)	Increase in body weight (g)
Control		106.2±4.83
Guan-mu-tong extract	1	107.9±4.13
	3	101.7±5.84
	9	98.4±2.93

3.2.2.2 Urinalysis

The urine of the treatment groups did not show significant differences from the control group in terms of color and amount of deposits.

3.2.2.2.1 Urine volume

Throughout the 3-month subchronic test, there was no significant changes in 24-hour total urine volume between the treatment groups and the control group (Figure 3.7).

3.2.2.2.2 Urinary protein

Within the 3-month duration, there was intermittently significant differences and much fluctuation in the total urinary protein collected in 24 hours between the treatment groups and the control group (Figure 3.8).

3.2.2.2.3 Urinary glucose

During the 3-month subchronic test, there was no significant differences of urinary glucose between the treatment groups and the control group (Figure 3.9).

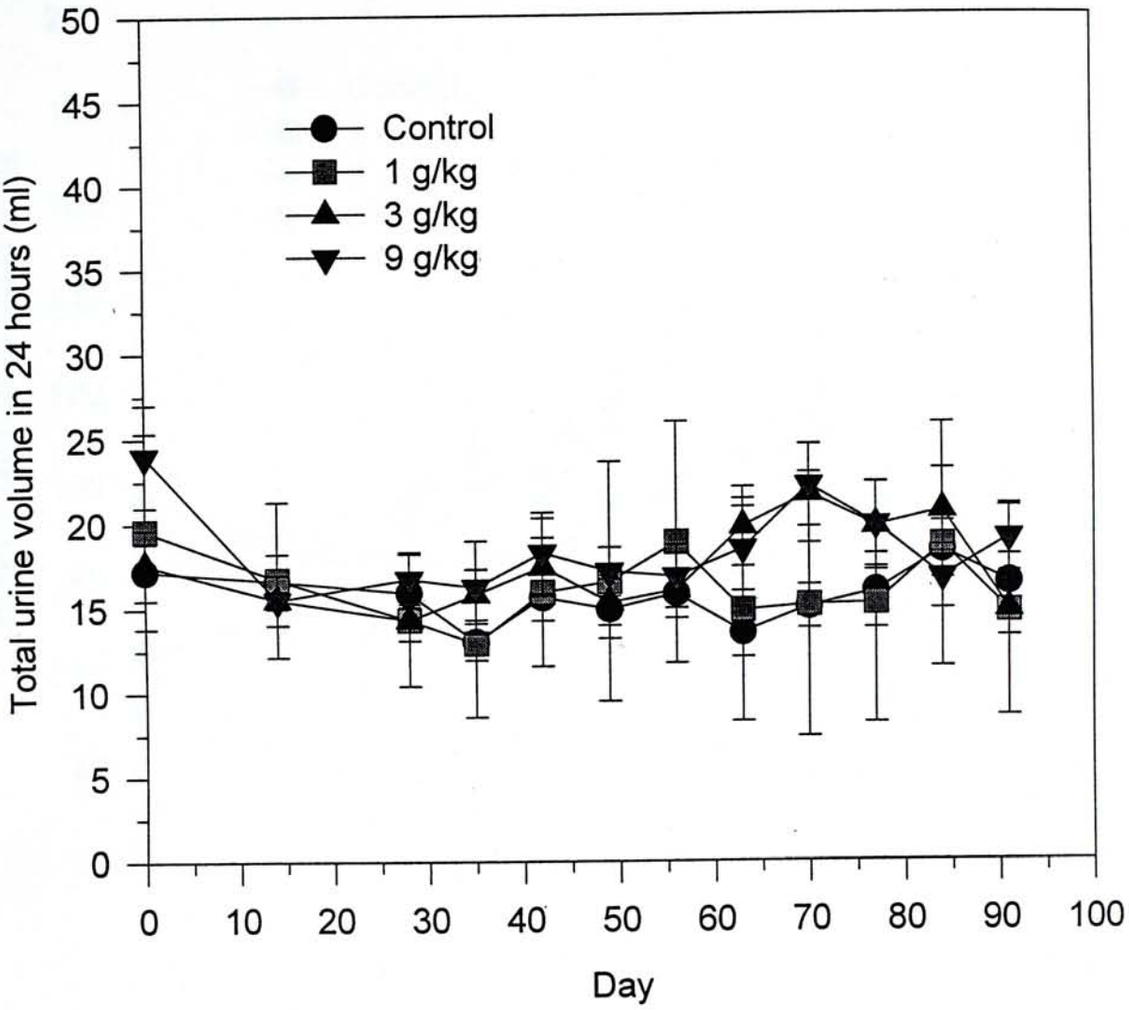


Figure 3.7: Effect of subchronic treatment of Guan-mu-tong on the urine volume of rats.

Values are expressed as arithmetic means ± standard errors.

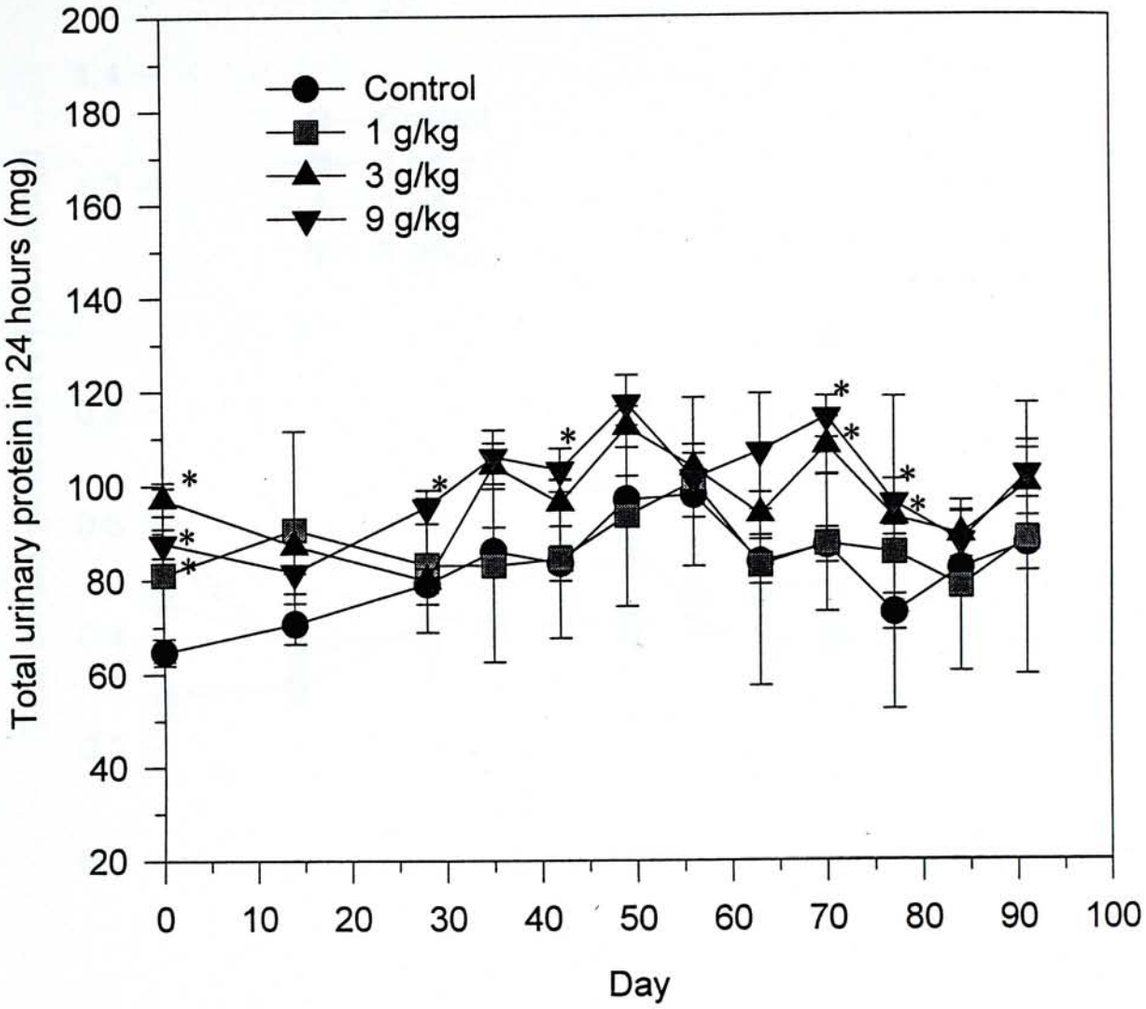


Figure 3.8 : Effect of subchronic treatment of Guan-mu-tong extract on the total urinary protein of rats.

Values are expressed as arithmetic means ± standard errors.
* Significantly different when compared with the control group, $p<0.05$.

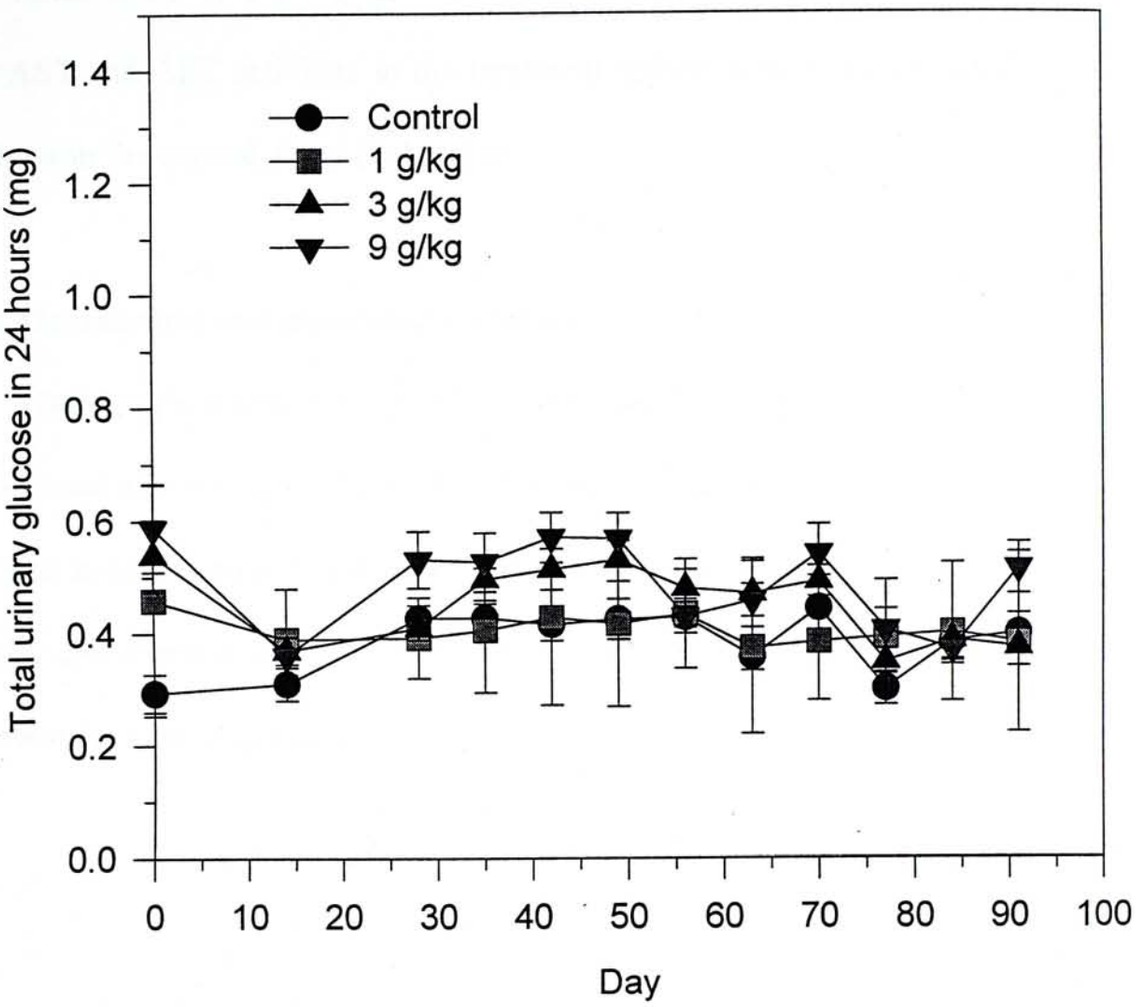


Figure 3.9: Effect of subchronic treatment of Guan-mu-tong extract on the total urinary glucose of rats.

Values are expressed as arithmetic means ± standard errors.

3.2.2.3 Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities

After the 3-month subchronic test of the aqueous extract Guan-mu-tong, the serum AST and ALT activities in the treatment groups were not significantly different from those in the control group (Table 3.6).

3.2.2.4 Macroscopic and microscopic findings

Macroscopic examination at the end of 3-month subchronic test of Guan-mu-tong did not reveal any lesions in the organs. Further microscopic study on kidney and liver showed no morphological changes as compared with the control group. Organ weight of the major and selected organs did not show significant differences between the treatment groups and the control group (Table 3.7).

Table 3.6: Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities of rats given Guan-mu-tong extract for 3 months. Values are expressed as arithmetic means ± standard errors.

Treatment group (n=10)	Dosage (g/kg i.g.)	AST activity (SF units/ml)	ALT activity (SF units/ml)
Control		169.82±17.96	105.82±3.13
Guan-mu-tong extract	1	176.27±12.78	135.69±6.66
	3	191.74±14.05	123.09±3.38
	9	180.66±19.75	116.00±7.06

Table 3.7: Vital organ weights of rats given Guan-mu-tong extract for 3 months. Values are expressed as arithmetic means ± standard errors .

Treatment group	Dosage	Body weight	Organ weight (in g) per 100 g body weight				
(n=10)	(g/kg i.g.)	(in g)	Heart	Liver	Spleen	Lung	Kidney
Control		306.60±8.02	0.324±0.006	3.411±0.045	0.163±0.006	0.430±0.008	0.734±0.012
Guan-mu-tong extract	1	318.30±7.33	0.318±0.005	3.507±0.046	0.171±0.005	0.433±0.016	0.732±0.016
	3	312.90±6.11	0.323±0.008	3.345±0.069	0.168±0.007	0.428±0.018	0.734±0.009
	9	299.10±4.02	0.321±0.005	3.323±0.086	0.181±0.005	0.435±0.008	0.731±0.010

Treatment group	Dosage	Organ weight (in g) per 100 g body weight	
(n=10)	(g/kg i.g.)	Stomach	Uterus
Control		0.530±0.015	0.167±0.008
Guan-mu-tong extract	1	0.519±0.011	0.153±0.008
	3	0.513±0.008	0.167±0.014
	9	0.534±0.015	0.147±0.013

3.2.3 Reproductive toxicity

The Guan-mu-tong extract exerted an anti-fertility effect in mice at high dosages: 60 and 90 g/kg i.g. The anti-fertility rates were 40 and 70% respectively. However, mortality was also resulted at the highest dosage; 20 % of the animals dosed at 90 g/kg i.g. died (Table 3.8).

At autopsies on PD_{16}, the numbers of implantation sites, normal fetuses, and corpus lutea were recorded. No significant differences in these parameters were found between the control and treatment groups (Table 3.8).

Table 3.8: Effect of Guan-mu-tong extract on the pregnancy rate, the fetus implantation, fetus formation and corpus luteum formation in mice sacrificed on PD_{16}.

Treatment group n=10	Dosage (g/kg i.g.)	No. of mice pregnant / animal dosed (anti-fertility rate)	No. of implantation sites per pregnant animal (arithmetic mean ± standard error)	No. of normal fetuses per pregnant animal (arithmetic mean ± standard error)	No. of corpus lutea per animal dosed (arithmetic mean ± standard error)
Control		10/10 (0%)	15.50±1.52	15.20±1.47	18.0±0.931
Guan-mu-tong extract	30 g/kg	10/10 (0%)	14.90±0.99	14.40±0.88	17.3±1.012
	60 g/kg	6/10 (40%)	14.00±0.82	13.33±1.02	15.6±0.930
	90 g/kg	3/8 [a] (70%)	16.00±1.53	13.33±2.91	16.0±1.304

[a] Two of the ten mice dosed died after dosing.

3.3 Toxicity of Ma-dou-ling

3.3.1 Acute Toxicity

After a single dose of (51.2-100 g/kg i.g.) the aqueous extract of Ma-dou-ling, toxic signs and mortality were observed on the following 21 days. Sedation was observed in mice of higher dosage groups immediately after dosing on the first day. However, most of them recovered with normal food consumption on the second day. Starting from the fourth to sixth days after dosing, there was onset of toxic signs in the morbid mice: sedation, piloerection, and tremors of the entire body during locomotion.

The body weight of the treatment groups was also affected. The control group had a larger gain in body weight (12.50±0.506 g) after 21-day observation while the survivors of the treatment groups had either smaller gain in body weight (3.14±1.079 for 51.2 g/kg i.g.) or even loss in body weight (3.80±1.114, 4.67±1.333 and 3.00±1.000 g for 64, 80 and 100 g/kg i.g. respectively) (Table 3.9). The body weight of the survivors in higher dosage groups dropped with time and did not regain their initial body weight by the end of the experiment even though the initial toxic signs had disappeared and their appetite restored again (Figure 3.10).

For those morbid animals, the body weight dropped rapidly with time several days before their death. Further toxic signs such as lack of body warmth, dyspnea, and apathy were observed. The mice died within 15 days and the death time depended on the dose. The death time was mainly on the third day to sixth day (Table 3.10). The LD_{50} with

95% confidence limit of male mice was found to be 64.74±1.22 g/kg i.g. and the dose-lethality curve was presented in Figure 3.11.

Autopsies of the survivors in the treatment groups did not show any gross abnormality or lesion in the major organs: heart, liver, spleen and lung except the kidney. Kidneys of all the treatment groups were pale yellow in color as compared with the dark brownish red color in the control group. The cut surface of the cortex was yellow and flabby while the pyramids and medulla were brick red in color.

Microscopic findings of the kidneys of the survivors in all the treatment groups showed massive tubular atrophy when compared with the control group (Figure 3.12–3.16). The epithelium of some proximal tubules showed vacuolation while some appeared flattened. Large irregular nuclei were found in tubular epithelial cells. The lumina of tubules were dilated and filled with desquamation of masses of cell debris and protein castes (Figure 3.14–3.16). Distal collecting tubules also showed marked changes with flattening of the tubules, occasional desquamation, and the presence of castes. The cytoplasm of most tubules showed a basophilic color (Figure 3.14–3.16).

Gross examination and histopathological studies were unable to be performed on mice that died intercurrently during the 21-day observation period due to cannibalism and advanced autolysis. Therefore, another batch of mice fed with the same dosages of the treatment groups were examined. The animals were sacrificed when the moribund signs appeared. The renal tissue sections from mice of 64 g/kg group showed massive tubular

necrosis with pyknotic nuclei throughout the inner portion of the cortex. The damages were predominantly the proximal straight tubules. In addition, packed glomeruli were observed (Figure 3.17 and 3.18).

Table 3.9: Change in body weight of surviving mice on 21st day after intragastric administration a single dose of Ma-dou-ling extract.

Group (n=10)	Dosage (g/kg i.g.)	No. of survivors per no. of mice dosed	Body weight gain in gram (arithmetic means ± standard errors)
Control		10 / 10	12.50±0.506
Ma-dou-ling extract	51.2	7 / 10	3.14±1.079***
	64	5 / 10	-3.80±1.114***
	80	3 / 10	-4.67±1.333***
	100	2 / 10	-3.00±1.000***

*** Significantly different when compared with the control group, $p < 0.001$.

Table 3.10: Death time of mice after intragastric administration of Ma-dou-ling extract.

Treatment of Ma-dou-ling extract (g/kg i.g.)	Death time after drug administration (day)							Total number of animals died
	3	6	9	12	15	18	21	
51.2	1	2	0	0	0	0	0	3
64	0	3	1	1	0	0	0	5
80	0	5	1	0	1	0	0	7
100	1	7	0	0	0	0	0	8

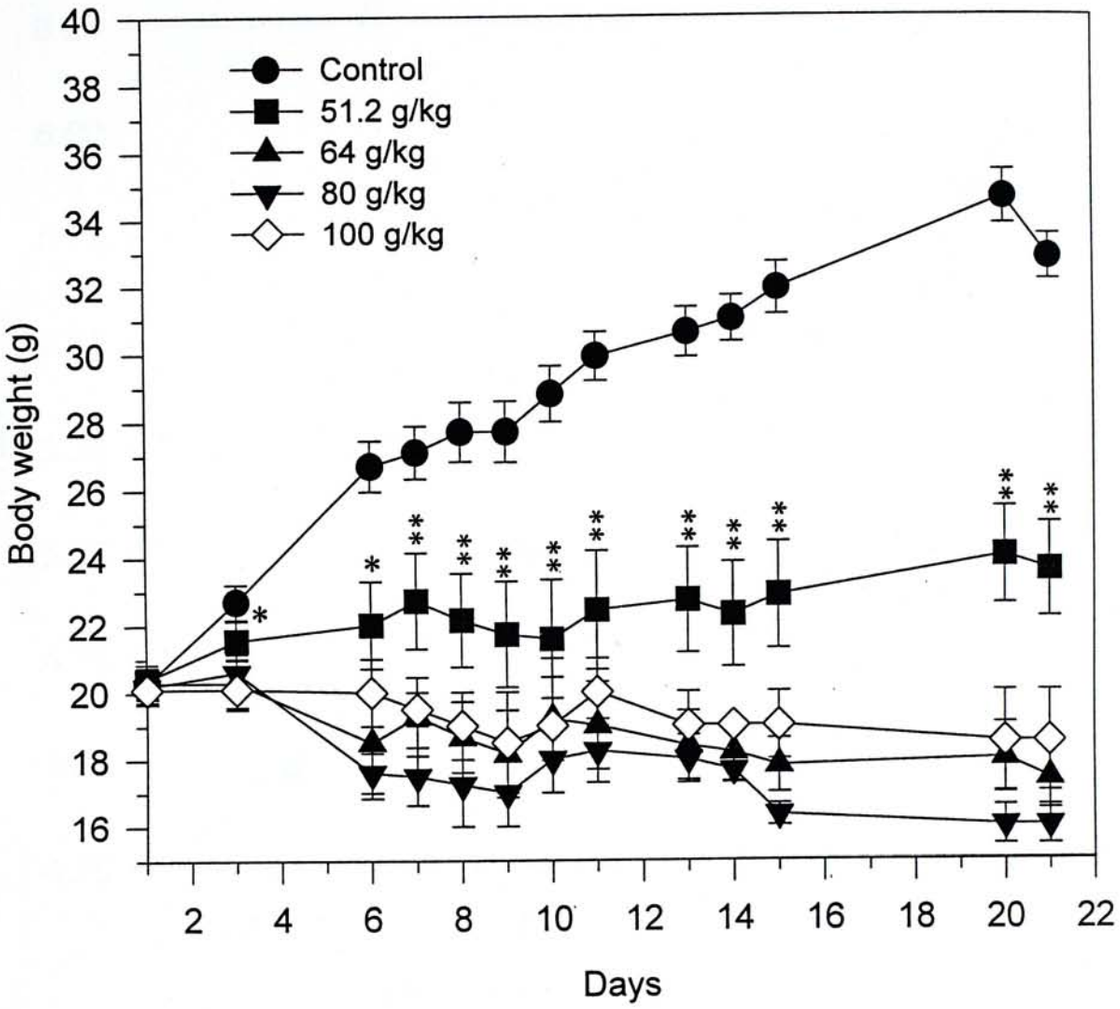


Figure 3.10: Effect of Ma-dou-ling extract on the body weight of surviving mice after acute treatment.

ICR mice in groups of ten were given a single dose of either distilled water (control group) or one of the four different dose levels of Ma-dou-ling extract (51.2, 64, 80 or 100 g/kg i.g).
Values are expressed as arithmetic means ± standard errors.

* All the values below are significantly different when compared with the control group, $p<0.05$.

** All the values below are significantly different when compared with the control group, $p<0.01$.

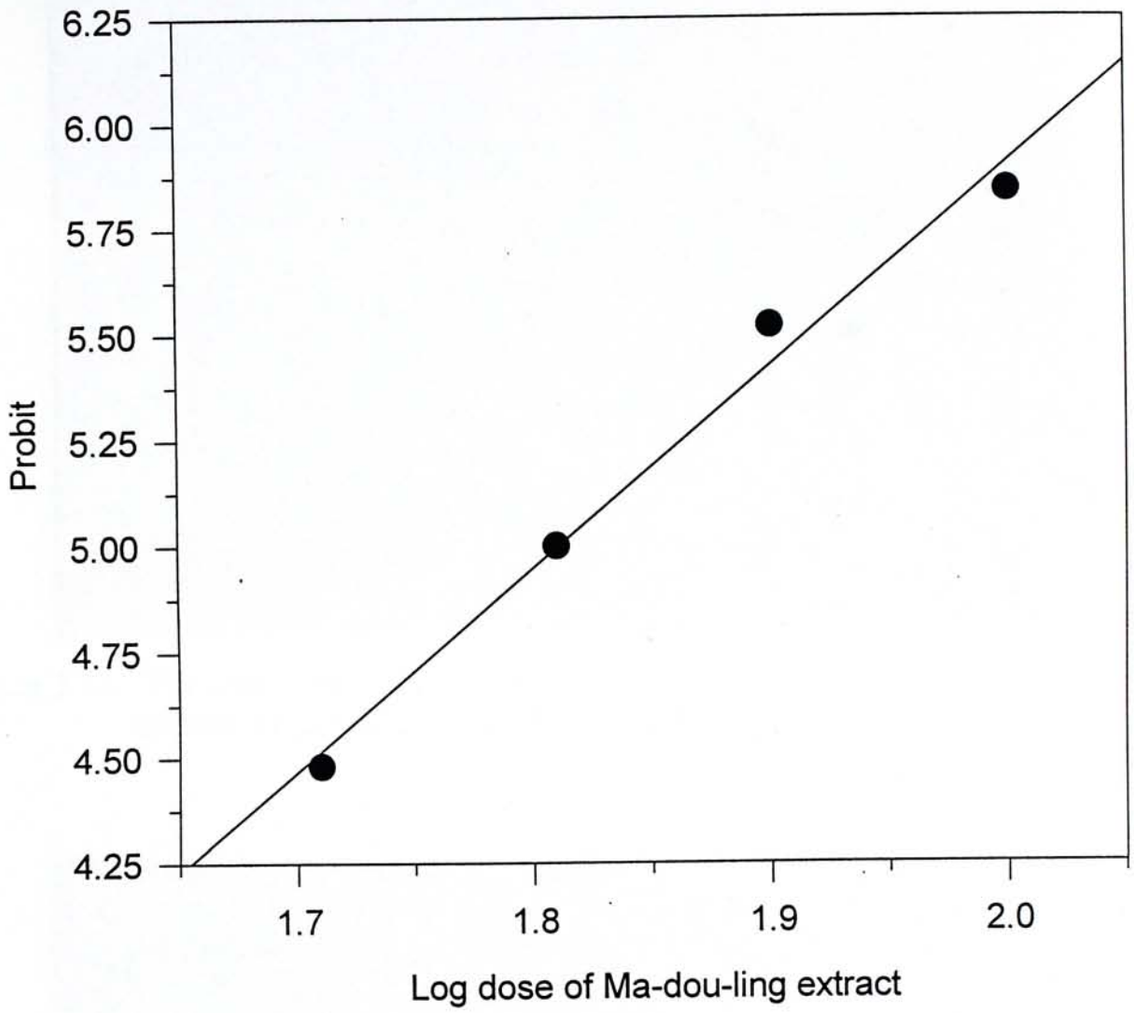


Figure 3.11: Dose-lethality curve of Ma-dou-ling extract on mice.

ICR mice in groups of ten were administered with a single dose of different dose levels of Ma-dou-ling extract (51.2-100 g/kg i.g.) LD_{50} in mice were found to be 64.74 ± 1.22 g/kg i.g.

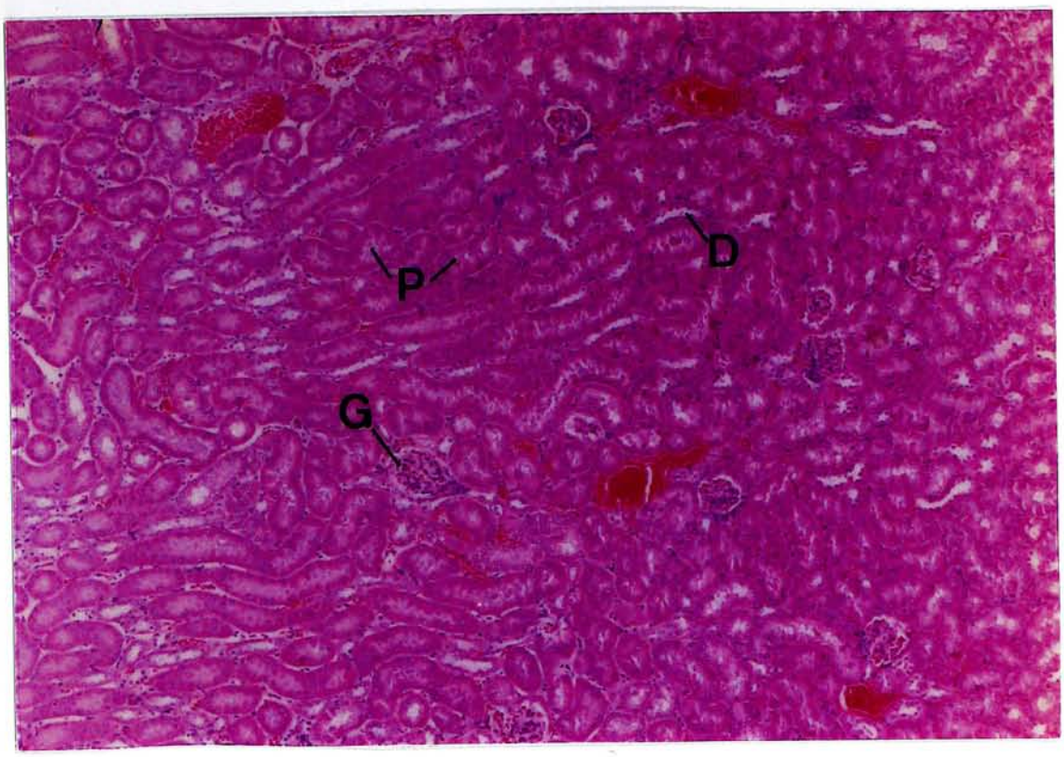


Figure 3.12: The renal cortex of mice in the control group. P, proximal tubules; D, distal tubules; G, glomerulus. H&E. Magnification x 48.

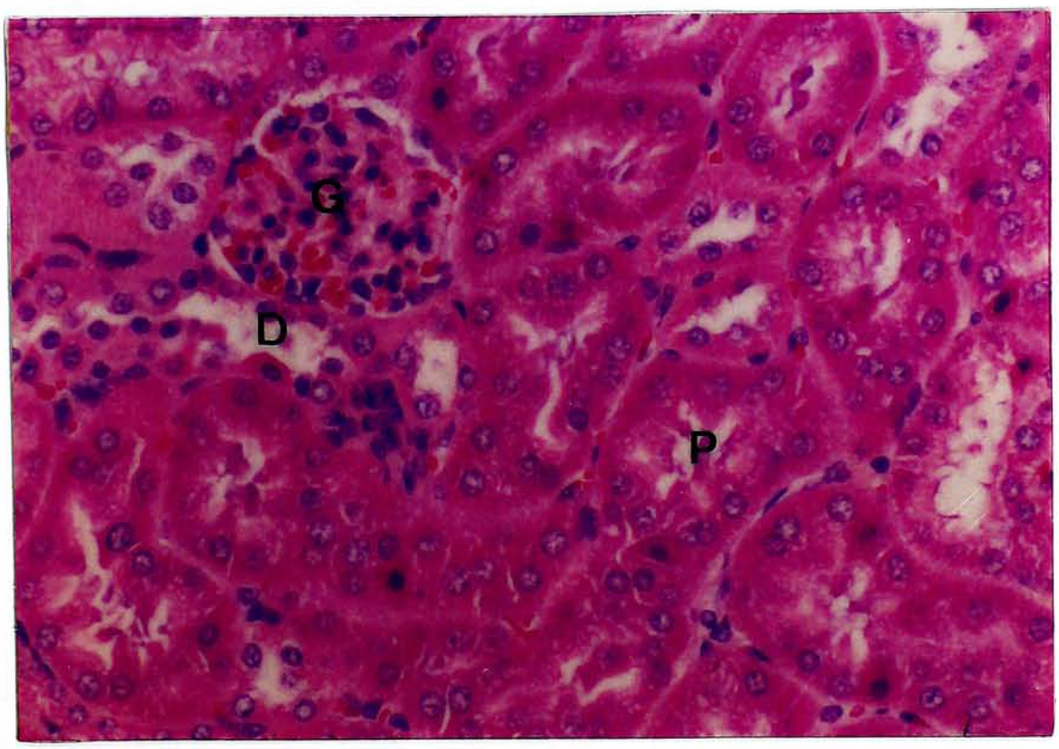


Figure 3.13: Normal proximal (P) and distal (D) tubules and glomerulus (G) of the renal cortex of mice in the control group. H&E. Magnification x 240.

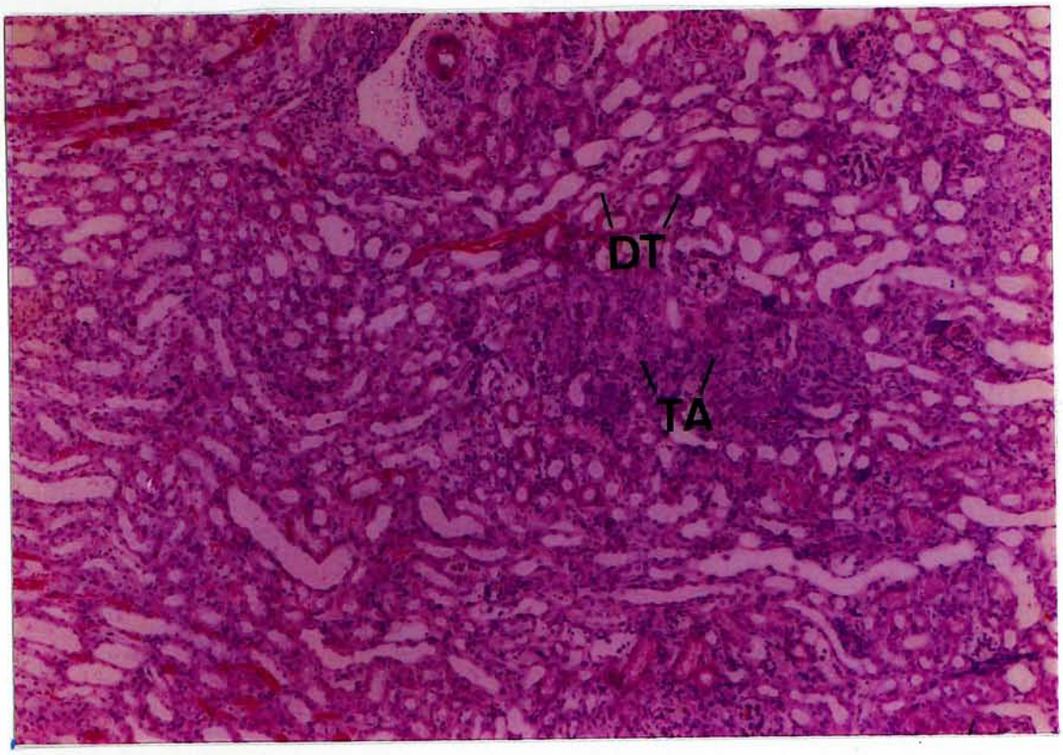


Figure 3.14: The renal cortex of surviving mice showed tubular atrophy (TA), dilated tubules (DT) on the 21st day after a single intragastric dose of 100 g/kg of Ma-dou-ling extract. H&E. Magnification x 48.

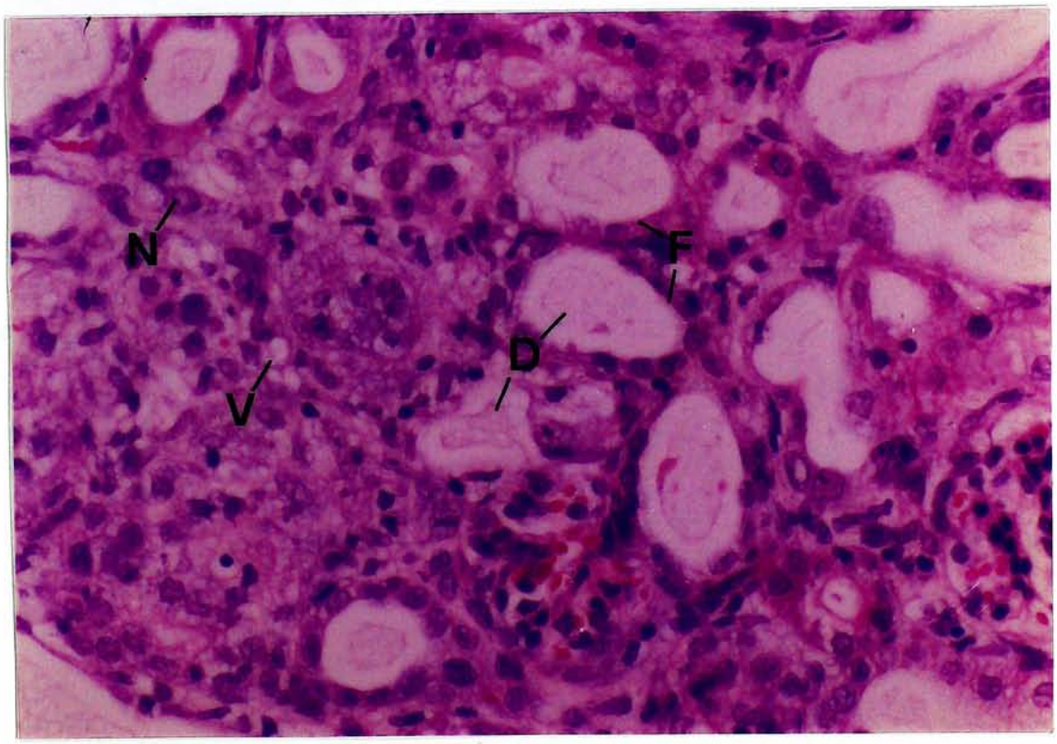


Figure 3.15: Flattening (F) and vacuolation (V) of tubules, large irregular nuclei (N), and desquamation of protein casts (D) in the renal cortex of surviving mice at the end of the 21-day observation period after a single intragastric dose of 100 g/kg of Ma-dou-ling extract. H&E. Magnification x 240.

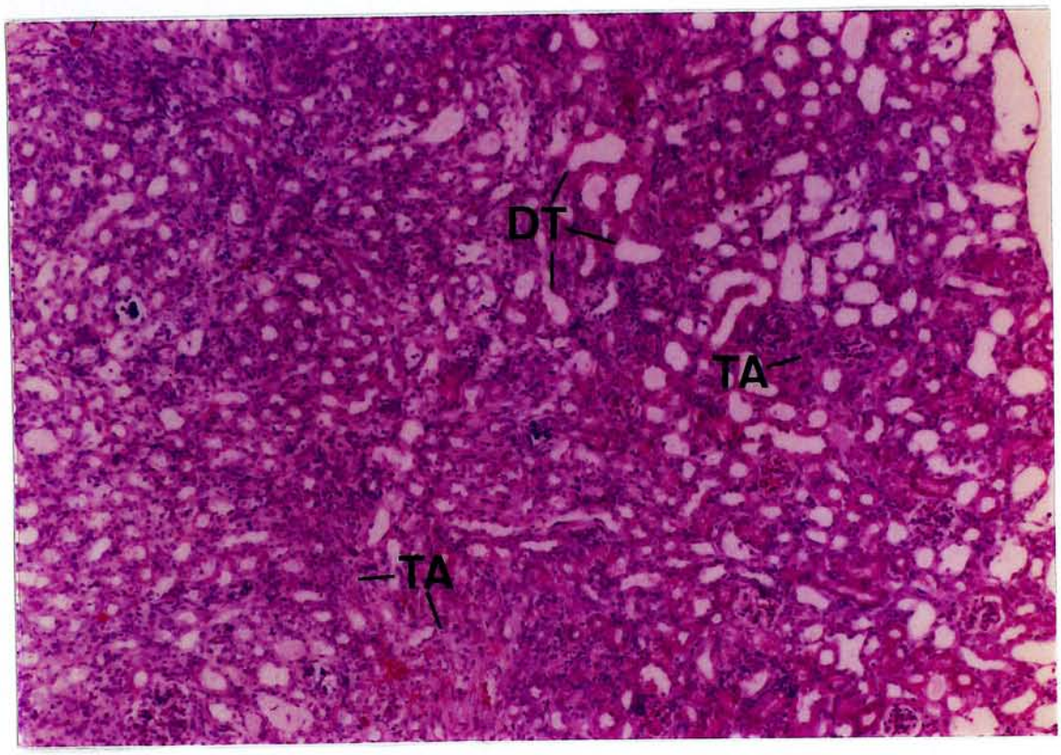


Figure 3.16: The renal cortex of mice showed tubular atrophy (TA), dilated tubules (DT) at the end of 21-day after a single intragastric dose of 51.2 g/kg of Ma-douling extract. H&E. Magnification x 48.

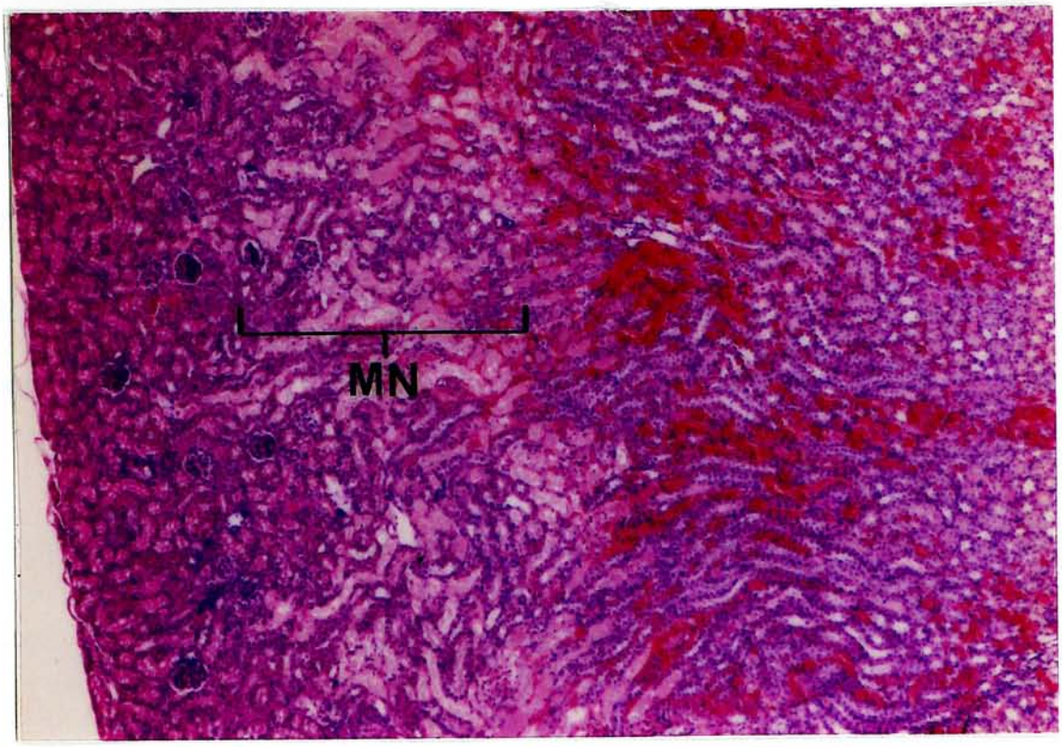


Figure 3.17: The kidney of moribund mice showed massive necrosis (MN) of the proximal tubules 5 days after a single intragastric dose of 64 g/kg of Madou-ling extract. H&E. Magnification x 48.

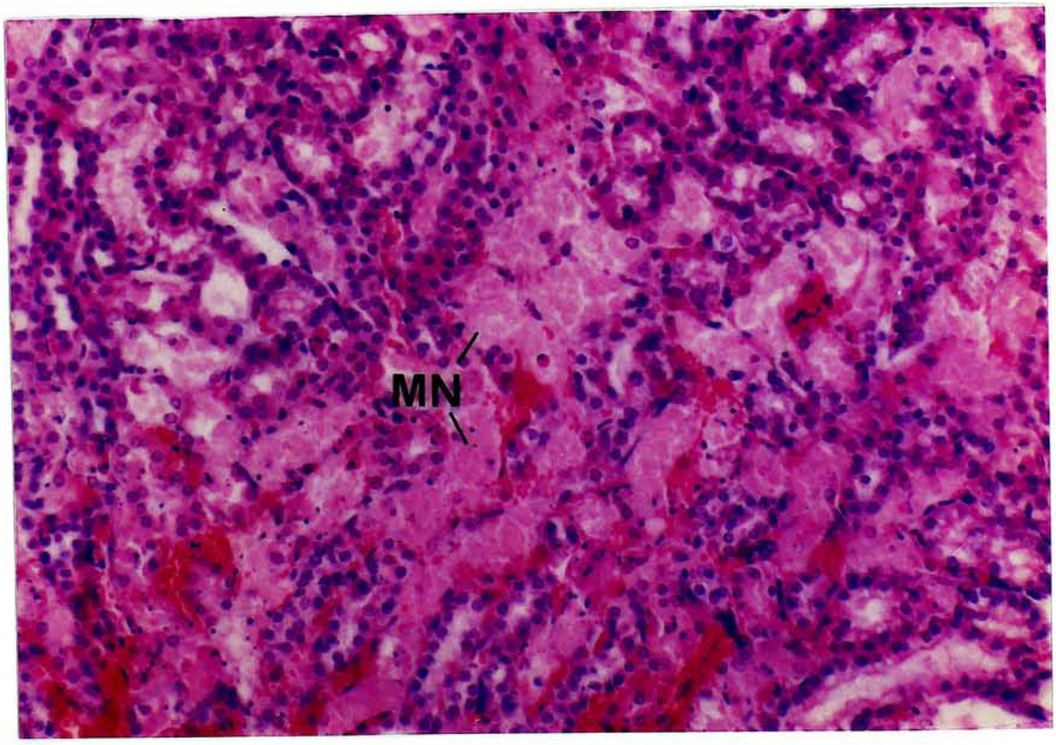


Figure 3.18: Massive necrosis (MN) of the proximal tubules of the moribund mice sacrificed on the 5th day after a single intragastric dose of 64 g/kg of Madou-ling extract. H&E. Magnification x 150.

3.3.2 Subchronic toxicity

The clinical state of rats in the treatment groups given aqueous extract of Ma-dou-ling extract 4 and 10 g/kg i.g. was not healthy. Piloerection and yellow hair was observed in these groups. Diarrhea was noted in the 10 g/kg group since one month after the onset of the experiment.

3.3.2.1 Body weight

The body weight of rats in the control and treatment groups increased gradually during the 3-month subchronic test. However, the rate of increase in body weight was much slower in all treatment groups. Since about the 70th day from the onset of the experiment, the 10g/kg i.g. group showed significant differences in body weight as compared with the control group (Figure 3.19). At the end of the 3-month test, gain in body weight of the 10 g/kg group was significantly lower than that of the control group (Table 3.11).

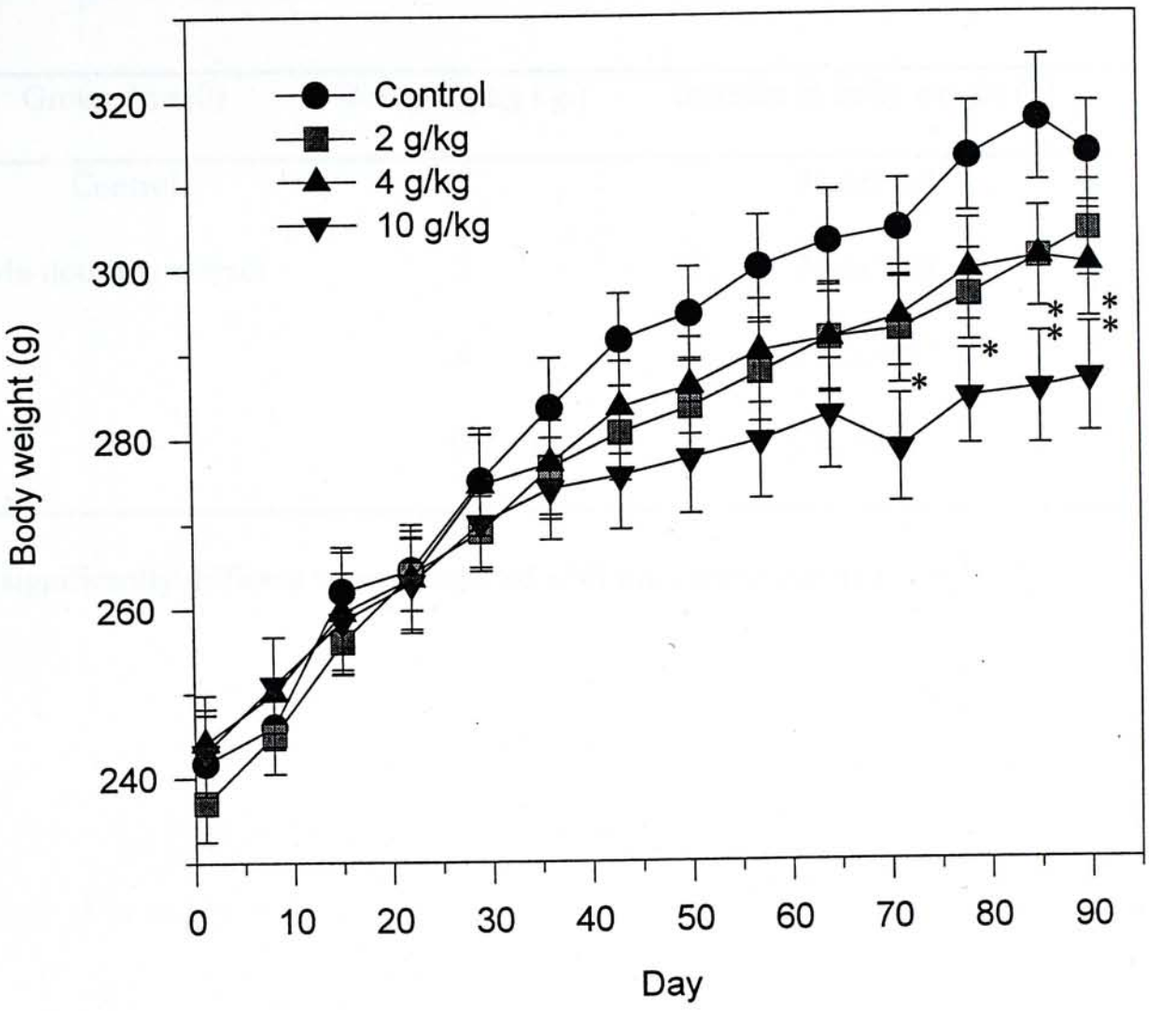


Figure 3.19: Effect of subchronic treatment of Ma-dou-ling extract on the body weight of rats.

Values are expressed as arithmetic means ± standard errors.

* Significantly different when compared with the control group, $p<0.05$.

** Significantly different when compared with the control group, $p<0.01$.

Table 3.11: Increase in body weight of rats after 3-month subchronic test with intragastric administration of Ma-dou-ling extract. Values are expressed as arithmetic means ± standard errors.

Group (n=10)	Dosage (g/kg i.g.)	Increase in body weight (g)
Control		90.6±6.68
Ma-dou-ling extract	2	70.8±7.02
	4	79.7±4.07
	10	63.4±4.81**

** significantly different when compared with the control group, $p<0.01$.

3.3.2.2 Urinalysis

Since two weeks after the onset of the intragastric administration of Ma-dou-ling extract, the urine color of rats in the 10 g/kg group was found to become dark brownish with lots of deposits. At a later period of time (one month after the start of the experiment), the urine of rats in the 4 g/kg group also showed the same color changes as in the 10 g/kg group.

3.3.2.2.1 Urine volume

During the 3-month subchronic test period, the total urine volume of all treatment groups showed an increasing trend. There was a significantly raised total urine volume in the 10 g/kg group in comparison with the control group at several time points towards the second half of the test period. At the end of 3 months, the total urine volume of all treatment groups was significantly elevated (Figure 3.20).

3.3.2.2.2 Urinary protein

The total urinary protein of the 10 g/kg group was significantly higher than that of the control group since day 1 of the subchronic test period. It increased with time with a final mean value of about 150 mg, almost 1-fold more than that of the control group (about 80 mg). Towards the end of the test, the 4 g/kg group also showed significantly raised total urinary protein as compared with the control group (Figure 3.21).

3.3.2.2.3 Urinary glucose

There was significantly elevated total urinary glucose in the 10 g/kg group since one week after the onset of the intragastric administration of Ma-dou-ling extract. It showed an increasing trend, and reached an amount of about 1.2 mg at the end of the test period, three times the amount as in the control group (about 0.4 mg). In addition, two weeks after the start of the experiment, the total urinary glucose in the 2 and 4 g/kg groups was significantly higher than that of the control group. Towards the end of the subchronic test, a significant increase in total urinary glucose was also found in the 4 g/kg group (Figure 3.22).

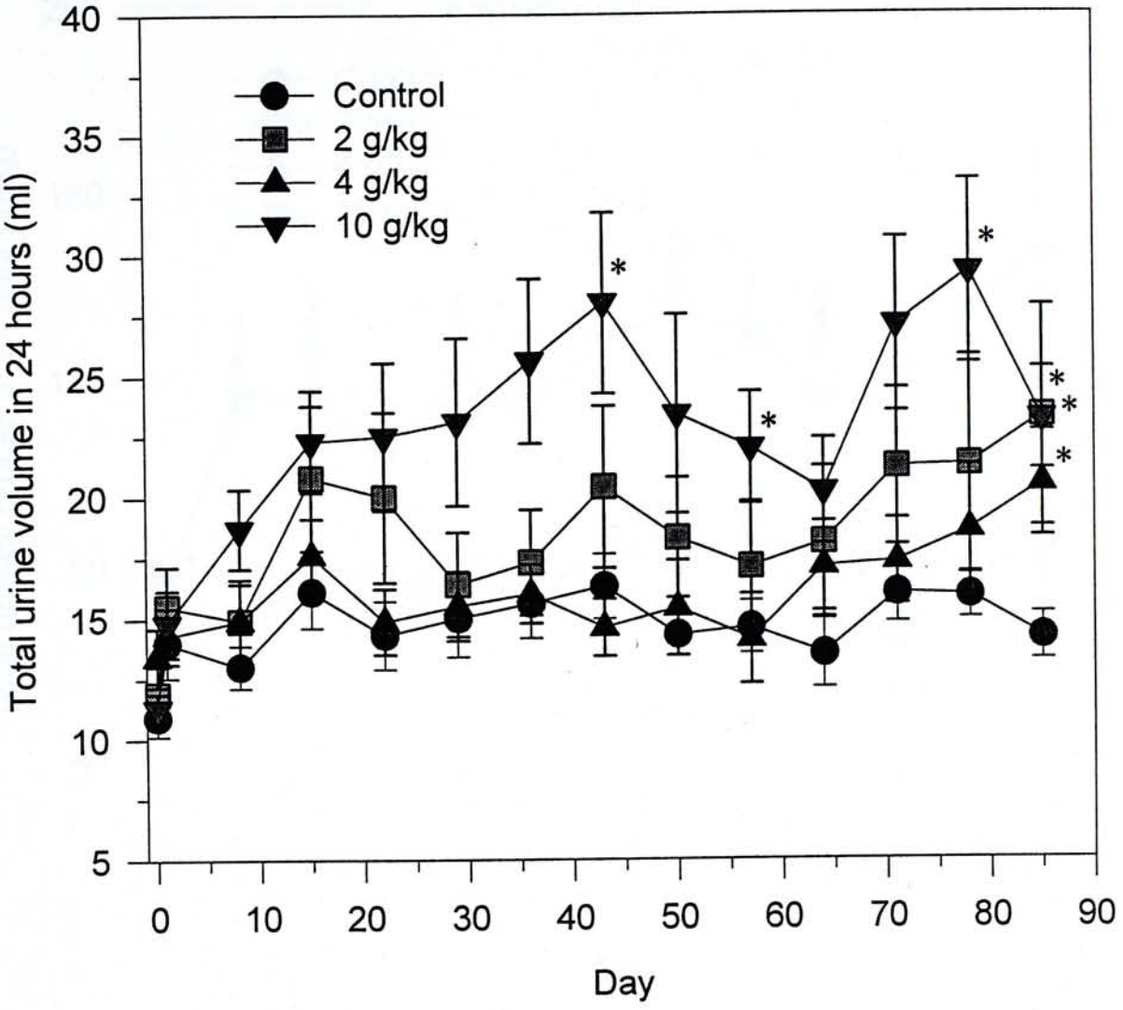


Figure 3.20: Effect of subchronic treatment of Ma-dou-ling extract on the urine volume of rats.

Values are expressed as arithmetic means ± standard errors.

* Significantly different when compared with the control group, $p<0.05$.

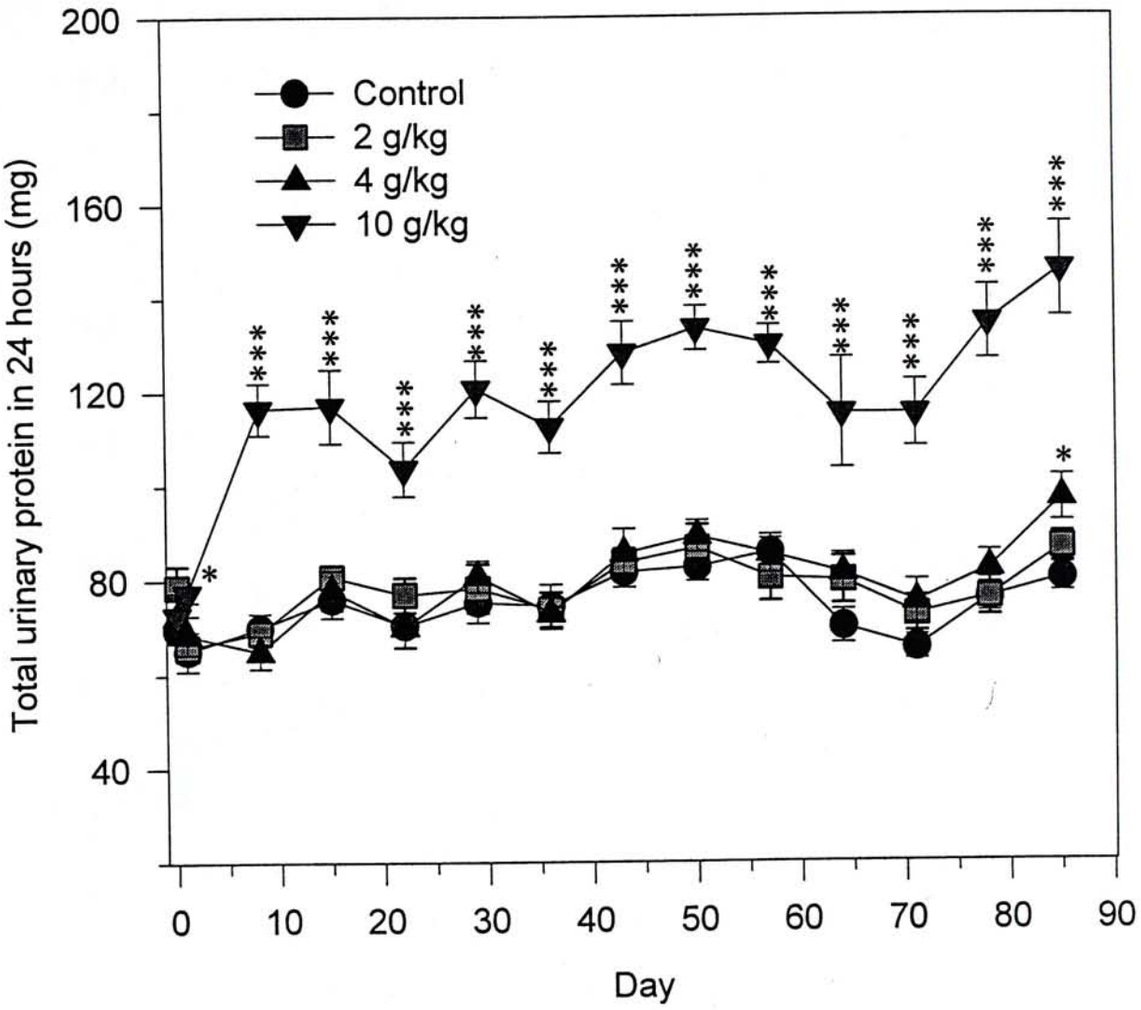


Figure 3.21: Effect of subchronic treatment of Ma-dou-ling extract on the total urinary protein of rats.

Values are expressed as arithmetic means ± standard errors.

* Significantly different when compared with the control group, $p<0.05$.

*** Significantly different when compared with the control group, $P<0.001$.

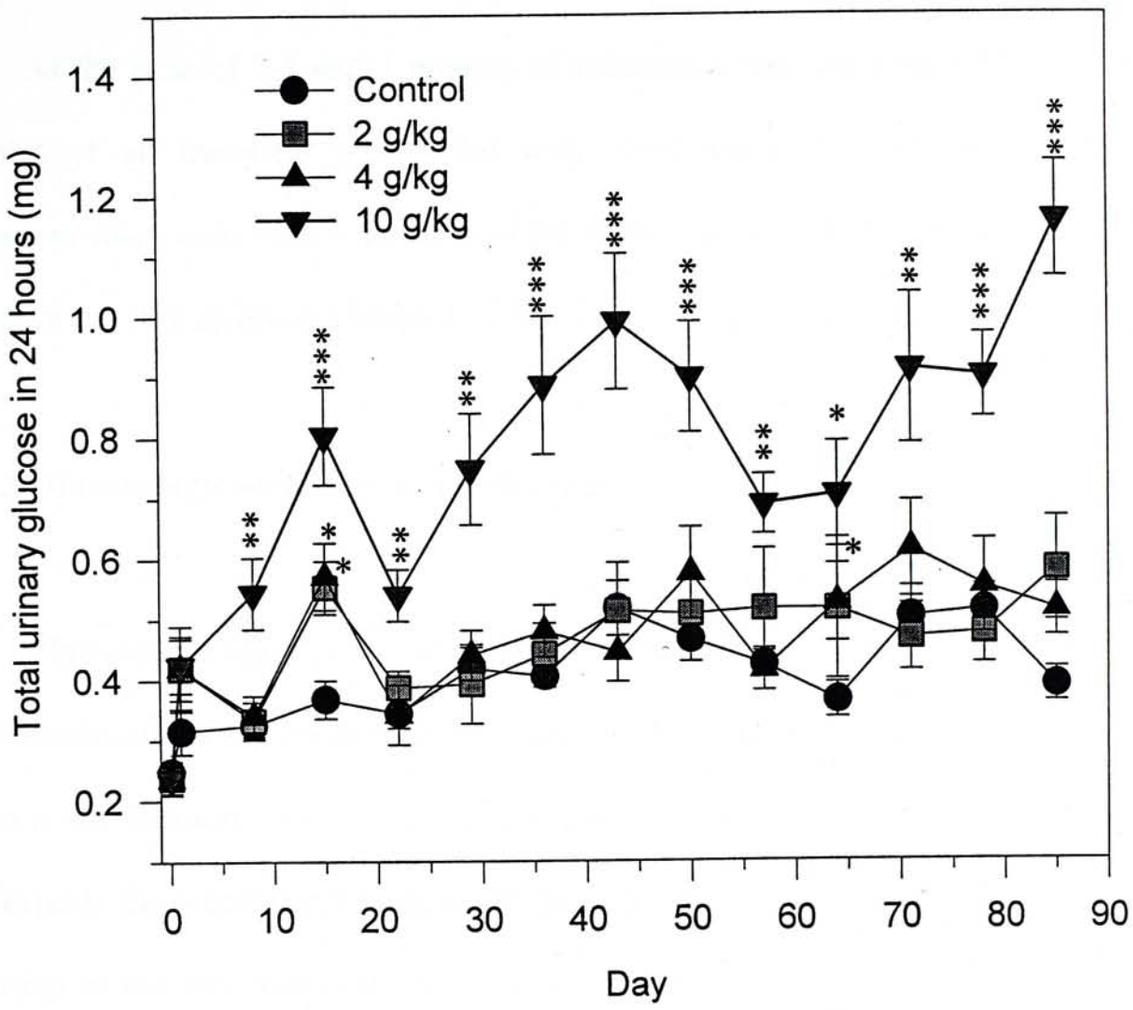


Figure 3.22: Effect of subchronic treatment of Ma-dou-ling extract on the total urinary glucose of rats.

Values are expressed as arithmetic means ± standard errors.

* Significantly different when compared with the control group, $p<0.05$.

** Significantly different when compared with the control group, $p<0.01$.

*** Significantly different when compared with the control group, $p<0.001$.

3.3.2.4 Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities

At the time of 1.5 and 3 months of subchronic test, the serum AST and ALT activities of all treatment groups fed with Ma-dou-ling did not show significant differences when compared with those of the control group except the increase in ALT activity in the 10 g/kg group (Tables 3.12 and 3.13).

3.3.2.3 Macroscopic and microscopic findings

Macroscopic examination of autopsies of rats fed with Ma-dou-ling extract at 1.5 and 3 months after the onset of the subchronic test revealed no visible lesion in the organs except in the stomach. At the mid-point of the 3-month subchronic test, papillomatosis was found in the forestomach in the 4 and 10 g/kg groups (Figure 3.23). The papillomas formation in the two treatment groups was found to be dose-dependent, with smaller nodular papilloma at the ridge between the forestomach and the body in the 4 g/kg group (Figure 3.24) and more severe and larger nodular papilloma covering the entire forestomach in the 10 g/kg group (Figure 3.25). Necropsies at the end of the 3-month test revealed qualitatively and quantitatively more severe lesion in the forestomach in all treatment groups (Figure 3.26). The whole forestomach of the 10 g/kg group was covered by papillomas. Forestomach ulceration was also noted in this group (Figure 3.27). The degree of papillomatosis in the 4 g/kg group was more severe at the end of the test than that found after 1.5-month treatment. More conspicuous papillmatosis was observed at

the later stage of the treatment period (Figure 3.28). The forestomach of the 2 g/kg group also showed lesion at the end of the 3-month subchronic treatment. It showed small nodules in contrast to the smooth surface in the control group (Figure 3.29 and 3.30).

Among the vital organ weight recorded at necropsies of rats at both 1.5- and 3-month after the onset of the test, significantly elevated stomach weight was noted in the 4 and 10 g/kg groups. In addition, liver, kidney and lung weight showed increases in the treatment groups (Tables 3.14 and 3.15).

Microscopic examination of the forestomach showed evidence of hyperkeratosis in all the treatment groups after 3-month subchronic treatment. Marked thick keratin layers were found in all the treatment groups as compared with the normal thin layers in the control group (Figure 31, 33, 34 and 37). In addition to hyperkeratosis, hyperplasia and carcinoma were also noted in the forestomach in the 4 and 10 g/kg group (Figure 31-33). There were irregular nuclei and vacuolated cytoplasm and numerous cell divisions in the squamous epithelial layer, which were different from the normal regular pattern in the control group (Figure 3.35 and 3.38). Dense leucocyte infiltration and signs of metastasis was also found (Figure 3.36).

In comparison with the control group, histological findings of the kidney and liver of all treatment groups after 1.5- and 3-month of treatment with Ma-dou-ling extract did not show evidence of lesion except some cloudy swelling found in cortical tubules of some animals in the 10 g/kg group.

Table 3.12: Serum level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities of rats given Ma-do-ling extract for 1.5 months. Values are expressed as means ± standard errors.

Treatment group (n=10)	Dosage (g/kg i.g.)	AST activity (SF units/ml)	ALT activity (SF units/ml)
Control		94.70±9.01	32.10±1.71
Ma-dou-ling extract	2	87.90±3.77	33.20±1.72
	4	86.10±2.02	34.50±1.04
	10	104.90±10.95	43.40±2.22***

*** Significantly different when compared with the control group, $p<0.001$.

Table 3.13: Serum level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities of rats given Ma-do-ling extract for 3 months. Values are expressed as arithmetic means ± standard errors.

Treatment group (n=10)	Dosage (g/kg i.g.)	AST activity (SF units/ml)	ALT activity (SF units/ml)
Control		101.81±5.80	40.91±1.32
Ma-dou-ling extract	2	117.29±4.54	46.87±2.16
	4	104.41±3.41	46.79±2.15
	10	100.09±6.33	51.80±2.23**

** Significantly different when compared with the control group, $p<0.01$.

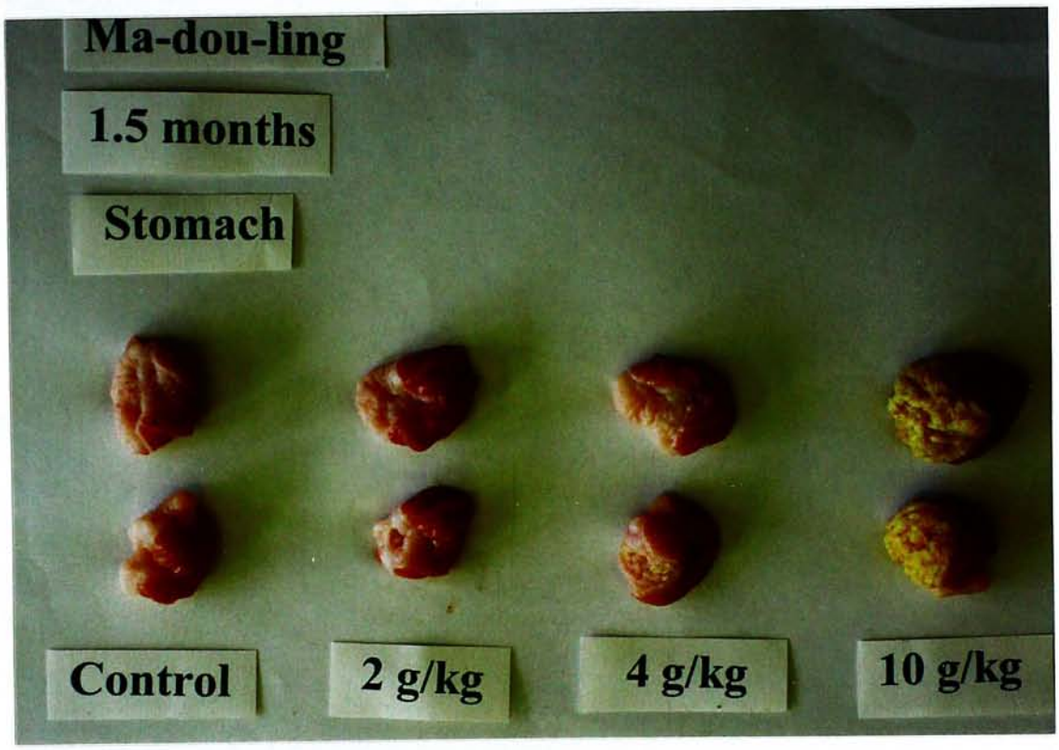


Figure 3.23: Rat forestomach of the control group and the treatment groups fed with Ma-dou-ling extract 2 g/kg, 4 g/kg and 10 g/kg for 1.5 months.

Figure 3.24: Smaller nodular papilloma at the ridge between the forestomach and the body of rat fed with Ma-dou-ling extract 4 g/kg for 1.5 months.

Figure 3.25: Nodular papilloma covered the entire forestomach without invading the body in rats fed with Ma-dou-ling extract 10 g/kg for 1.5 months.

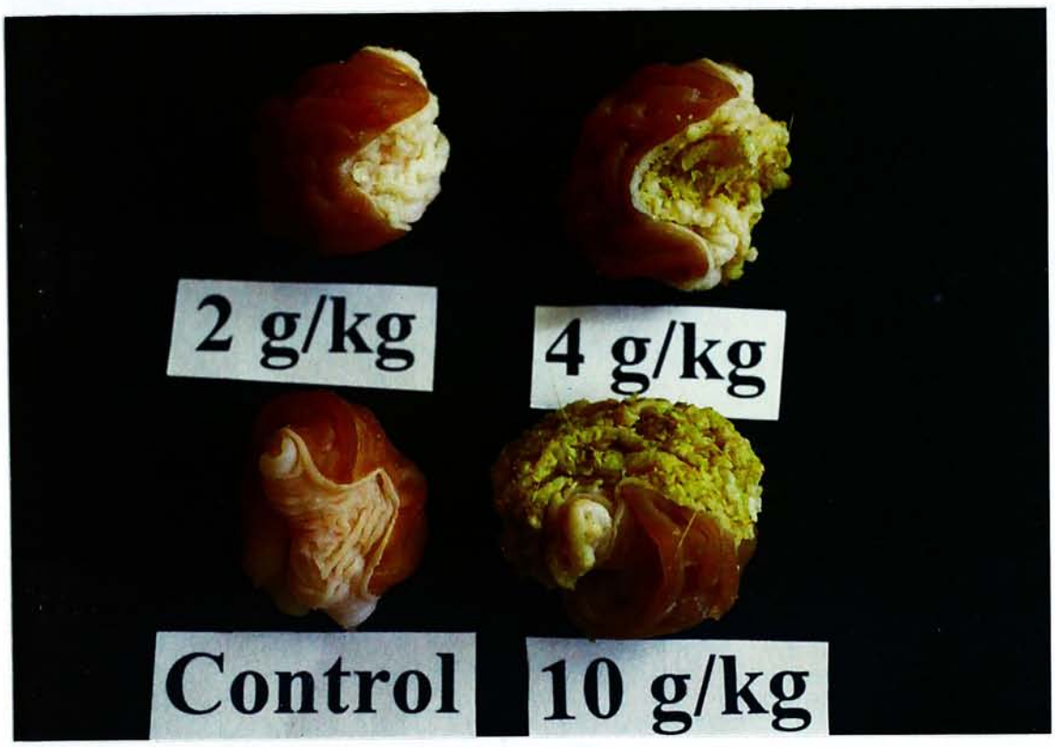


Figure 3.26: Rat forestomach of the control group, and the treatment groups fed with Madou-ling extract 2, 4 and 10 g/kg for 3 months showing dose-dependent papillomas formation.

Figure 3.27: The entire rat forestomach of the treatment group fed with Ma-dou-ling extract 10 g/kg for 3 months, showing multiple papillmas and ulceration.

Figure 3.28: Papillomas of the forestomach of the treatment groups fed with Ma-dou-ling extract 4 g/kg for 3 months.

Figure 3.29: Small nodules on the forestomach of the treatment group fed with Ma-douling extract 2 g/kg for 3 months.

Figure 3.30: Normal forestomach with smooth surface in the control group fed with vehicle (water) for 3 months.

Table 3.14: Vital organ weights of rats given Ma-dou-ling extract for 1.5 months. Values are expressed as arithmetic means ± standard errors.

Treatment group (n=10)	Dosage (g/kg i.g.)	Body weight (in g)	Organ weight (in g) per 100 g body weight				
			Heart	Liver	Spleen	Lung	Kidney
Control		284.80±6.17	0.333±.0.007	3.506±0.073	0.183±0.005	0.444±0.014	0.694±0.016
Ma-dou-ling extract	2	282.60±7.35	0.327±0.007	3.301±0.035	0.186±0.008	0.453±0.012	0.679±0.018
	4	271.60±5.06	0.326±0.008	3.460±0.057	0.189±.0006	0.464±0.015	0.711±0.011
	10	261.05±7.83	0.329±0.003	3.796±0.101*	0.190±0.006	0.492±0.012	0.753±0.019*

Treatment group (n=10)	Dosage (g/kg i.g.)	Organ weight (in g) per 100 g body weight	
		Stomach	Uterus
Control		0.483±0.014	0.197±0.020
Ma-dou-ling extract	2	0.489±0.014	0.181±0.011
	4	0.559±0.016*	0.184±0.013
	10	0.811±0.046***	0.179±0.013

* Significantly different when compared with the control group, $p < 0.05$.

*** Significantly different when compared with the control group, $p < 0.001$.

Table 3.15: Vital organ weights of rats given Ma-dou-ling extract for 3 months. Values are expressed as arithmetic means ± standard errors.

Treatment group (n=10)	Dosage (g/kg i.g.)	Body weight (in g)	Organ weight (in g) per 100 g body weight				
			Heart	Liver	Spleen	Lung	Kidney
Control		313.50±5.97	0.314±0.006	3.233±0.096	0.177±0.006	0.410±0.010	0.679±0.026
Ma-dou-ling extract	2	304.30±5.73	0.314±0.004	3.304±0.071	0.191±0.009	0.450±0.005**	0.682±0.008
	4	300.20±6.42	0.323±0.005	3.204±0.086	0.178±0.007	0.416±0.011	0.752±0.025*
	10	286.70±6.45*	0.306±0.007	3.474±0.085	0.188±0.007	0.476±0.013**	0.733±0.017

Treatment group (n=10)	Dosage (g/kg i.g.)	Organ weight (in g) per 100 g body weight	
		Stomach	Uterus
Control		0.562±0.012	0.174±0.012
Ma-dou-ling extract	2	0.600±0.014	0.172±0.011
	4	0.698±0.018***	0.196±0.017
	10	1.039±0.028***	0.168±0.012

* Significantly different when compared with the control group, $p < 0.05$.

** Significantly different when compared with the control group, $p < 0.01$.

*** Significantly different when compared with the control group, $p < 0.001$.

Figure 3.31: Forestomach of the treatment group fed with Ma-dou-ling extract 10 g/kg for 3 months, showing hyperkeratosis (HK) and hyperplasia (HP) in the squamous stratified epithelium. H&E. Magnification x 48.

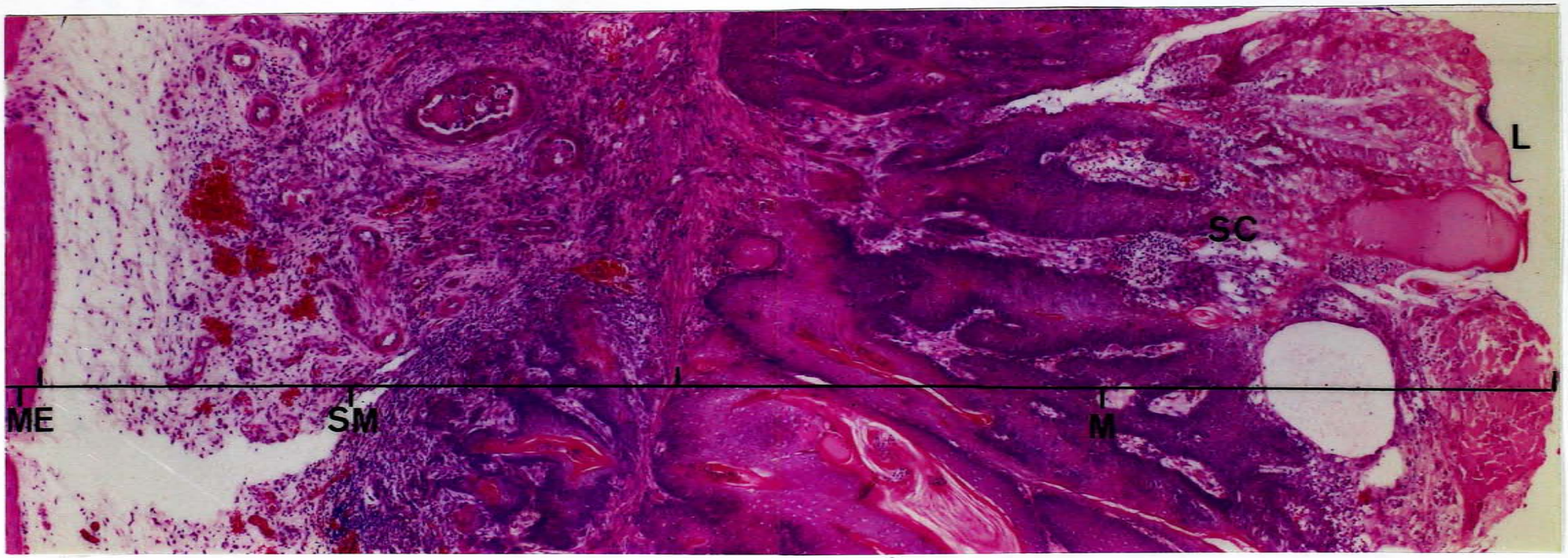


Figure 3.32: Forestomach of the treatment group fed with Ma-dou-ling extract 10 g/kg for 3 months, showing squamous carcinomas (SC). L, luminal side ;M, mucosa; SM, submucosa; ME, muscularis externa. H&E. Magnification x 48.

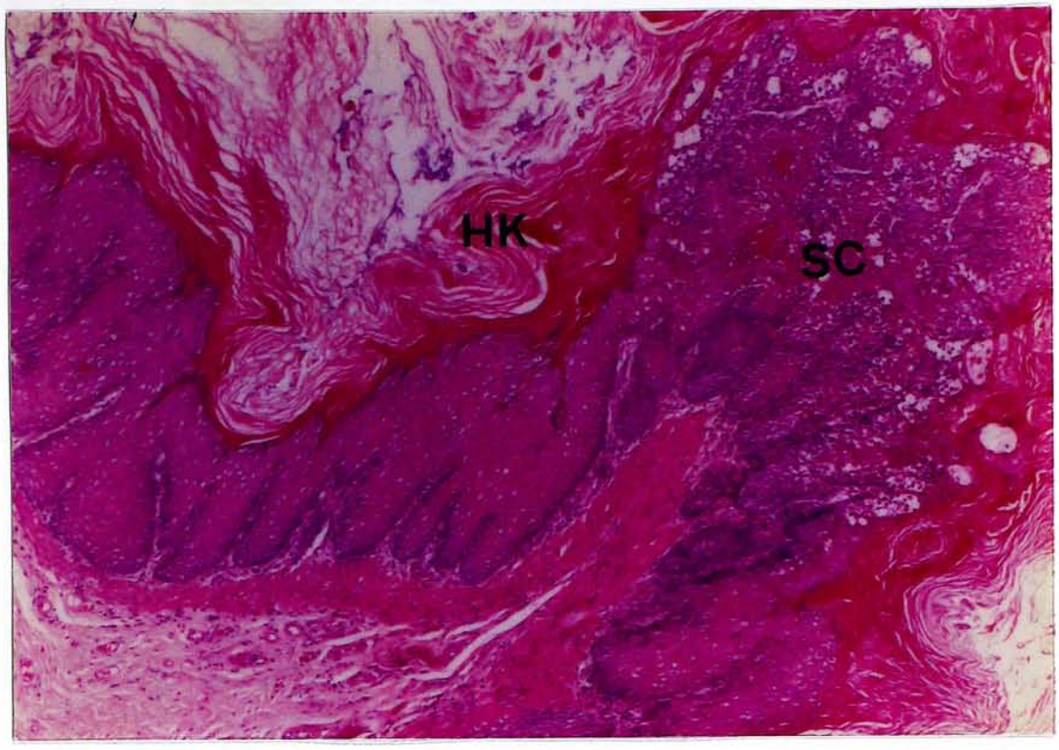


Figure 3.33: Forestomach of the treatment group fed with Ma-dou-ling extract 4 g/kg for 3 months, showing squamous carcinomas (SC) with hyperkeratosis (HK). H&E. Magnification x 48.

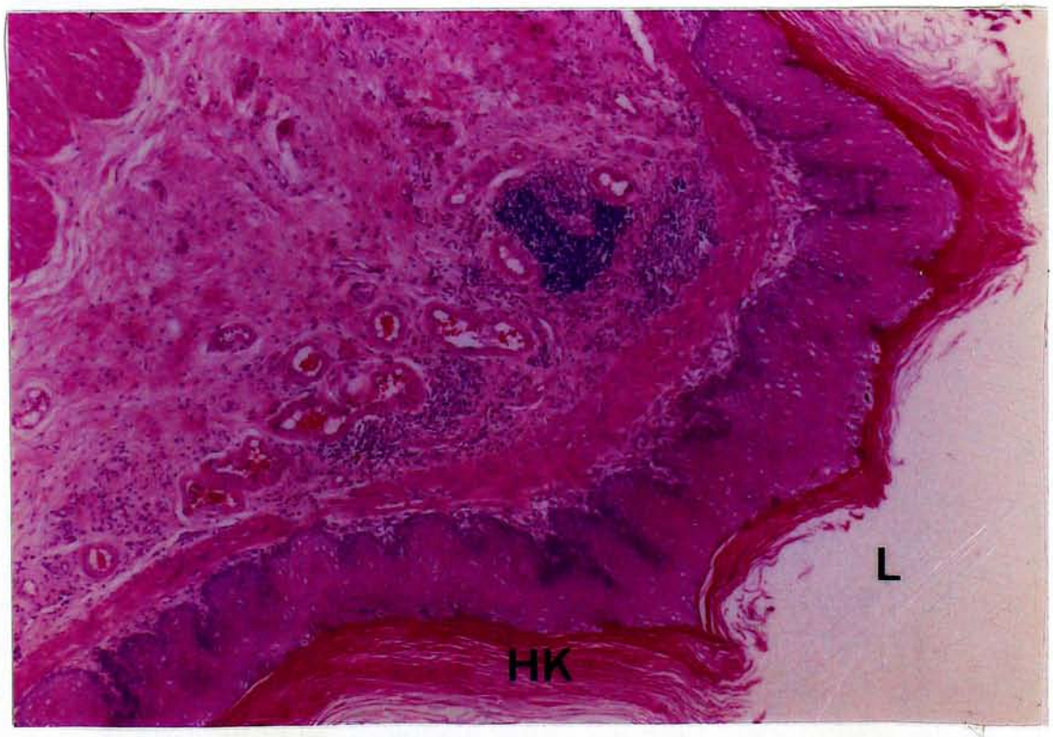


Figure 3.34: Forestomach of the treatment group fed with Ma-dou-ling extract 2 g/kg for 3 months, showing hyperkeratosis (HK). L, luminal side. H&E. Magnification x 48.

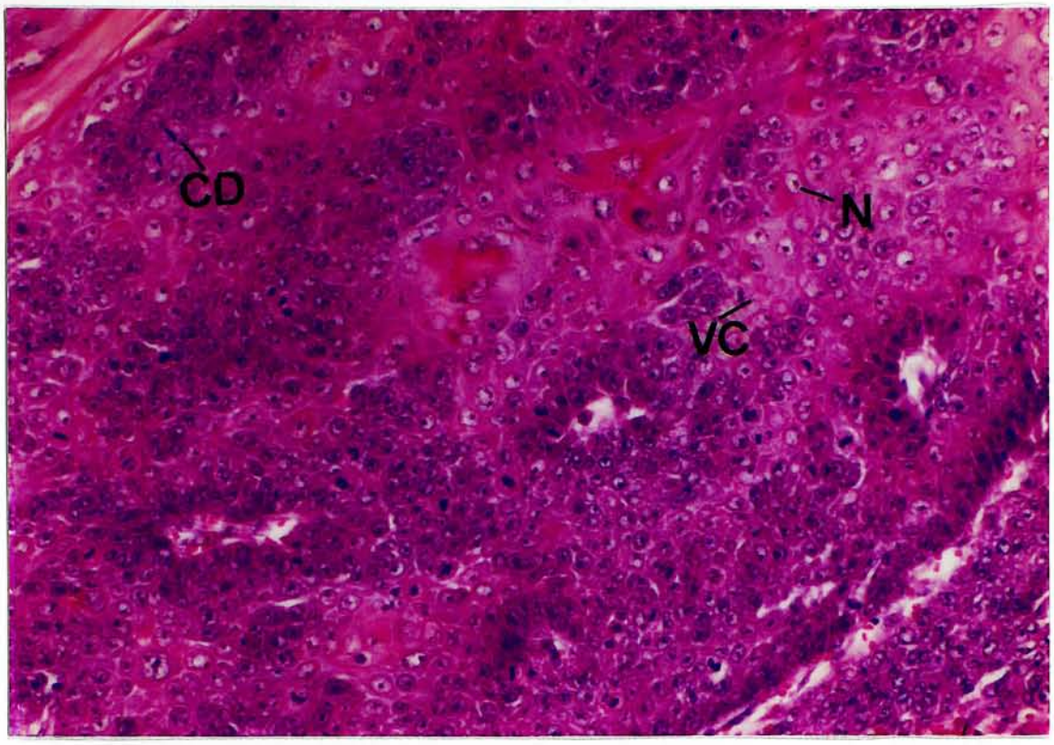


Figure 3.35: Vacuolated cytoplasm (VC), irregular nuclei (N) and cell divisions (CD) in the squamous carcinoma of the treatment group fed with Ma-dou-ling extract 10g/kg group after 3 months. H&E. Magnification x 150.

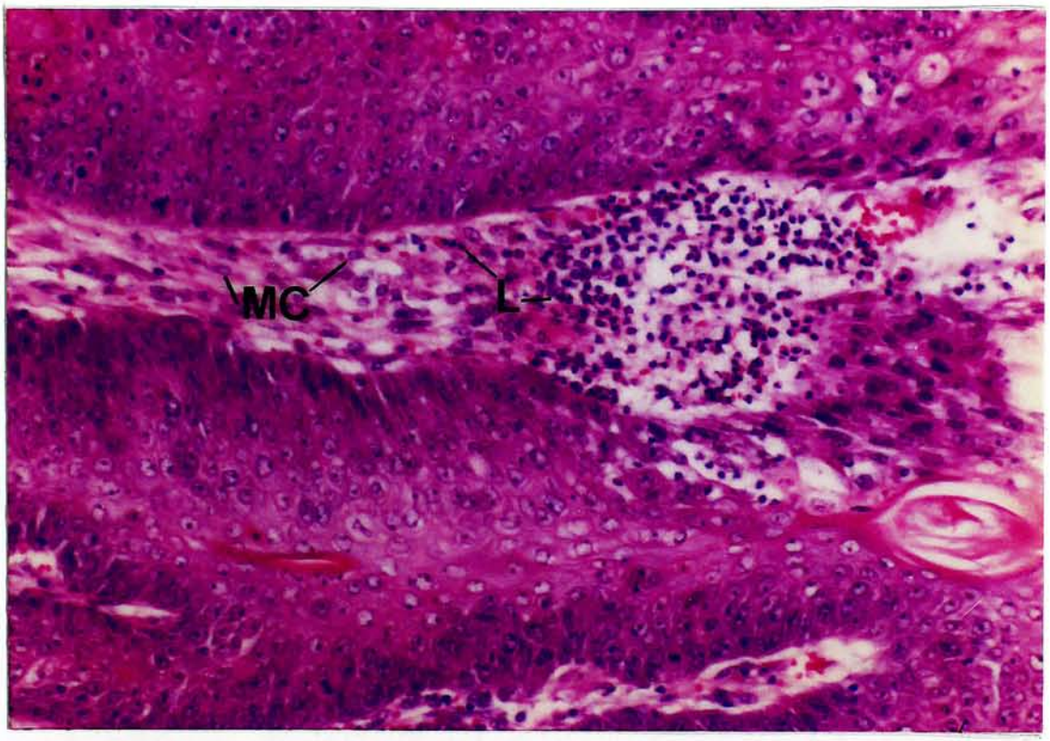


Figure 3.36: Metastasis and massive leucocytes infiltration in the forestomach squamous epithelium in the treatment group fed with Ma-dou-ling extract 10 g/kg for 3 months. The figure showing aggregate of leucocytes (L) and the metastasizing malignant cells (MC) in the blood stream. The malignant cells with irregular cell size and shape. H&E. Magnification x 150.

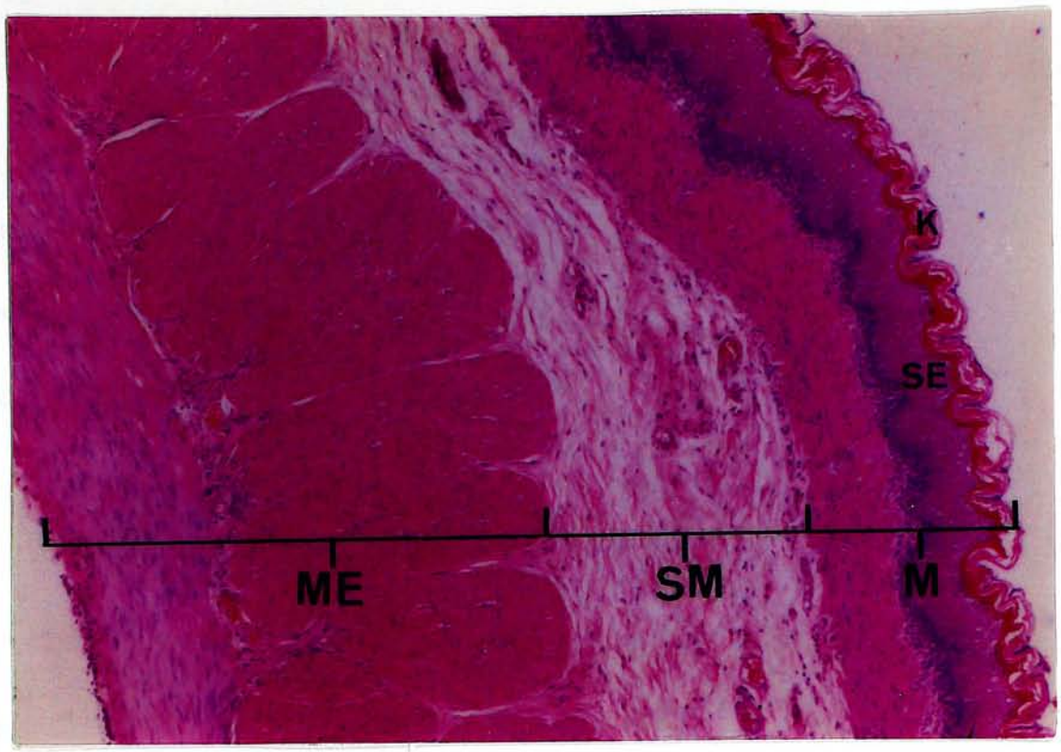


Figure 3.37: Rat forestomach of the control group at the end of the 3-month subchronic toxicity test, showing normal squamous epithelial layer (SE) with thin layer of keratin (K). M, mucosa; SM, submucosa; ME, muscularis externa. H&E. Magnification x 48.

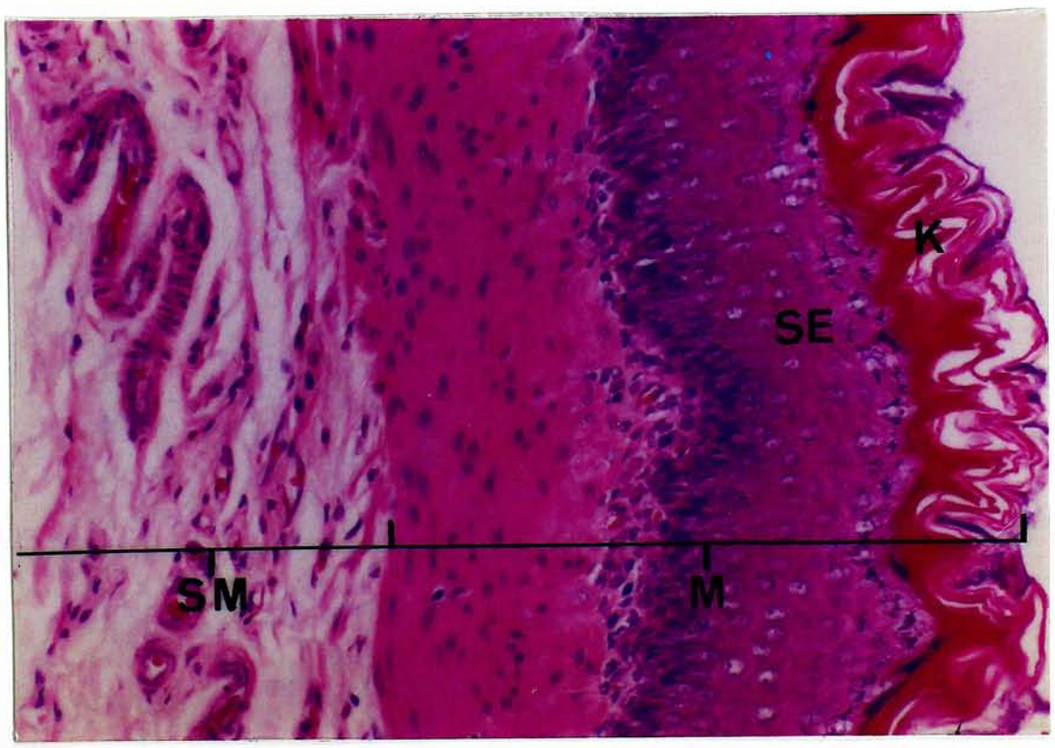


Figure 3.38: Rat forestomach of the control group at the end of the 3-month subchronic toxicity test, showing normal, regular pattern of the squamous epithelial cells (SE) with uniform cell size and shape. K, keratin; M, mucosa; SM, submucosa. H&E. Magnification x 150.

3.3.3 Reproductive toxicity

The Ma-dou-ling extract at 4.15–32 g/kg i.g. showed dose-related anti-fertility activity. At higher dosages of 19.2 g/kg i.g. and above, 100% anti-fertility rate was observed. Moreover, 40% of mortality were resulted in the treatment group fed with the extract 32 g/kg i.g. (Table 3.16).

At autopsies on PD_{16}, the numbers of implantation sites, normal fetuses and corpus lutea were recorded. No significance differences in these parameters were found between the control and treatment groups (Table 3.16).

Table 3.16: Effect of Ma-dou-ling extract on the pregnancy, fetus implantation, fetuses formation and corpus lutea formation in mice.

Treatment group (n=10)	Dosage (g/kg i.g.)	No. of mice pregnant / animal dosed (anti-fertility rate)	No. of implantation sites per pregnant animal (arithmetic mean ± standard error)	No. of normal fetuses per pregnant animal (arithmetic mean ± standard error)	No. of corpus lutea per animal dosed (arithmetic mean ± standard error)
Control		10/10 (0%)	14.40±0.542	13.80±0.663	15.8±0.490
Ma-dou-ling extract	4.15 g/kg	8/10 (20%)	16.13±0.934	14.00±1.282	16.4±0.618
	6.91 g/kg	8/10 (20%)	17.63±0.706	15.38±1.017	17.9±0.690
	11.52 g/kg	2/10 (80%)	16.50±2.500	14.00±1.000	15.8±0.696
	19.20 g/kg	0/10 (100%)	–[b]	–[b]	16.9±.0605
	32.00 g/kg	0/6[a] (100%)	–[b]	–[b]	14.5±0.719

[a] four of ten mice died after dosing.

[b] all mice were not pregnant at time of autopsies.

Chapter Four: Discussion

4.1 Chemical analysis

4.1.1 Thin layer chromatography

The two bands observed in the reference mixture of AA should be AA-I and AA-II based on the information provided by the supplier. Therefore the two herbal medicines of *Aristolochia* species examined in TLC analysis contained these two compounds. These herbal medicines were then subjected to a more accurate qualitative and quantitative analysis performed by HPLC.

4.1.2 High performance liquid chromatography

AA is the active component found in most of the *Aristolochia* species. HPLC was used to determine the AA contents in Guan-mu-tong and Ma-dou-ling qualitatively and quantitatively. Among the two herbal medicines studied, the crude drug Guan-mu-tong was found to contain a higher total AA content than Ma-dou-ling.

Since Chinese herbal formulas are traditionally prepared by decocting herbs with water, experimental animals in the present toxicity studies were fed with decoction of the two herbs. Thus, the hot water extracts of Guan-mu-tong and Ma-dou-ling were also analyzed for their AA contents. In the preparation of aqueous extract for the toxicity studies on animals, Guan-mu-tong, which was presented in thin slices, was extracted

with water in the weight to water volume ratio of 1:11 while Ma-dou-ling was extracted in 1:30. The different extraction methods were used because Guan-mu-tong was first studied and its volume and water absorption extent was much smaller than that of Ma-dou-ling. Ma-dou-ling which was studied later had a greater volume and absorbed much water during the extraction process, and therefore a larger weight to water volume ratio was selected. Thus, a decoction of the same weight to water volume ratio of Guan-mu-tong (1:11) and Ma-dou-ling (1:30) used in toxicity studies was prepared for HPLC analysis.

The quantitative analysis revealed that the aqueous extract of Ma-dou-ling had a higher AA content than that of Guan-mu-tong. About 50 % of the AA content in Ma-dou-ling was extracted by the aqueous extraction process. On the other hand, a large amount of AA in Guan-mu-tong was not extracted and still remained in the crude drug after decocting. The possible reason for the lower amount of AA extracted from Guan-mu-tong than from Ma-dou-ling is the different surface area of the two herbal drug available for extraction. Ma-dou-ling in form of a dry fruit was crushed into pieces before decocting, while Guan-mu-tong was in form of thin slices from the supplier. As a result, the increased surface area to volume ratio of Ma-dou-ling sample would enable AA to dissolve in water, while the smaller surface area to volume ratio of Guan-mu-tong sample would limit the leakage of AA into water. This difference in the amounts of AA extractable from the two herbs would probably reflect the actual conditions of decocting in their normal use in Chinese medicine.

4.2 Toxicity of Guan-mu-tong

4.2.1 Acute toxicity

In the present study, the highest possible dosage of Guan-mu-tong extract at 337.72 g/kg i.g. was given to the mice by gavage. After a 21-day observation period, only 4 of 10 mice dosed died. The dose applied was extremely high but still only 40 % of the animals died. Thus, the lethal dose of Guan-mu-tong extract could not be definitely determined but was higher than 337.72 g/kg i.g.

4.2.2 Subchronic toxicity

From the results of subchronic toxicity, the Guan-mu-tong extract did not show any significant toxic effect. There was no abnormality in physical observation in the 3 months of the test period. No significant change in body weight was found. Urinalysis did not show any effect of Guan-mu-tong extract on the renal functions though there was an intermittent increase in urinary protein found in the group fed with Guan-mu-tong extract 9 g/kg i.g. As there was difference in random sampling at the beginning of the study, it is not clear if the intermittent increase in urinary protein was an indication of renal impairment. For the study of serum enzymatic activities, serum AST and ALT activities in the treatment groups revealed no changes. Gross and histological examination also showed no distinct lesion when compared with the control group.

Guan-mu-tong contains nephrotoxic mixture, aristolochic acid (AA). From the analysis of TLC and HPLC, the crude drug Guan-mu-tong contains a high AA content. However, a relatively large reduction in AA content was found in the Guan-mu-tong extract. The reduction may account for the absence of the impairment of renal function, which is a known toxic effect of AA (Mengs and Stotzem, 1992), found in the subchronic toxicity. In conclusion, the Guan-mu-tong extract at dosage up to 9 g/kg did not show any toxic effect on female rats in the subchronic toxicity test.

4.2.3 Reproductive toxicity

In the present study on reproductive toxicity of Guan-mu-tong, an anti-fertility activity could be observed only at relatively high dosages (60–90 g/kg i.g.) in mice. In addition, at the highest dosage (90 g/kg i.g.) tested in this study, only 70% anti-fertility rate was recorded.

Guan-mu-tong extract did not reduce the number of implantation sites in all the treatment groups. Besides, visual inspection of the fetuses did not show any abnormal morphological appearance.

Although the ethnomedical literature indicated that Guan-mu-tong is contraindicated, the present study showed that the antifertility activity of Guan-mu-tong extract became obvious only at very high dosages.

4.3 Toxicity of Ma-dou-ling

4.3.1 Acute toxicity

The gross examination and histopathological findings indicated that the primary toxic target organ of Ma-dou-ling extract was the kidney. The renal tissues of mice in all experimental groups showed tubular impairment of different severity, depending on the dosages given.

In the present study, kidneys were obtained from moribund mice several days before death. Macroscopically, the cortex was pale yellow in color as compared to the contrasting brick red color of the medulla. Microscopically, massive tubular necrosis was noted in the inner cortex and outer strip of outer medulla. The straight part of the proximal tubules was the predominant impaired portion. This specific target portion, together with the characteristic macroscopic appearance of the kidney indicated that the animals suffered from renal failure due to acute tubular necrosis (Darmady and MacIver, 1980; Solez, 1993).

Acute tubular necrosis is a potentially reversible decrease in renal functions following injury to the kidneys. The massive tubular necrosis was irreversible in a short period of time (several days) but were able to recover in a long-term period (7–10 days) if the animals could survive the challenge. Thus, those animals that could not tolerate the injury for the first few days would succumb to death (Walmsley and White, 1983). Acute tubular necrosis consists of four sequential phases: initiation, oliguric/anuric, early

diuretic, and late diuretic phases. The initiation phase is the period following the toxic exposure and refers to the time interval between the injury and the onset of acute renal failure. This explains the reasons of the gradual development of toxic signs and the late onset of death time in the acute toxicity of Ma-dou-ling extract on mice. The oliguric/anuric phase is marked by a reduced urine output containing cellular granular casts and rises in blood urea, creatinine, potassium and phosphate levels. This phase usually lasts a few days to several weeks. Reparation of the impaired tubules starts in the early and late diuretic phases. The early diuretic phase is characterized by excessive urinary output because of the impaired concentrating ability of the kidney. Restoration of tubular functions and concentrating ability occurs in the late diuretic phase (Williams and Mallick, 1994). The process of restoration of renal functions and reparation of tubular tissue was demonstrated by the microscopic findings in renal tissues of the surviving mice sacrificed on the 21st day after dosing. Cell debris of necrotic cells from proximal tubules, flattening and cytoplasmic vacuolation of tubular epithelial cells, and the dilation of the tubular lumina were found. In addition, basophilic cytoplasm and large irregular hyperchromatic nuclei were noted in the tubular epithelial cells (Solez, 1993). All these changes indicated tubular regeneration and the ongoing process of restoration of renal functions in the surviving animals. On the other hand, in severe injury, the moribund animals were unable to withstand the intoxication and died during the oliguric/anuric phase.

Since the proximal tubules, especially the straight portion, was found to have massive tubular necrosis in the early period of the injury (at about day 5–6 after dosing

for the moribund animals), it is possible that this straight portion (known as S_3 segment) was the main and primary target tissue. One of the main functions of the proximal straight tubules is to actively secrete organic ions such as weak acids or bases and harmful compounds into the tubular lumen. Transport of organic solutes and the metabolism of toxins by tubular cells results in high concentration of toxins and generation of toxic metabolites in tubular cells. Therefore, the straight portion is particularly susceptible to damages (Swan and Bennett, 1993; Valtin and Schafer, 1995).

The comparison of the acute toxicity of Ma-dou-ling extract and that of AA reported in literature (Mengs, 1987; Mengs and Stotzem, 1993) showed similarity in several aspects particularly on the macroscopic and microscopic findings of damage on renal tissues. In addition, experimental animals given either agents had similar delay in death time after dosing. Thus, AA may be one of the toxins, if not the only, in Ma-dou-ling extract causing the toxic effects in acute toxicity test on mice.

4.3.2 Subchronic toxicity

During the 3-month subchronic toxicity test with Ma-dou-ling extract, there was a reduction in body weight gain in all treatment groups. Certain adverse reactions in the treated animals could be the causes of the reduction in body weight gain. Diarrhea was found in the group fed with Ma-dou-ling extract 10 g/kg. The Ma-dou-ling extract given may be irritant to gastrointestinal tract, causing a faster rate of peristalsis for eliminating the toxicants. Thus, the digestion and absorption of ingested food would be hampered.

Another cause of reduction in the body weight gain might be the impairment in renal function caused by Ma-dou-ling extract. Proteinuria and glucosuria was found in the treatment groups. The continual loss of essential body constituents - protein and glucose - in urine impeded the growth of the animals.

The purpose of urinalysis in this study was to examine the effect of Ma-dou-ling extract on renal functions. The present study showed that subchronic administration of Ma-dou-ling extract caused impaired renal functions in rats, resulting in proteinuria, glucosuria and polyuria. The severity demonstrated a dose- and time-dependent relationship.

Glucosuria usually occurs either in diabetes mellitus (systemic disease) or primary renal disease. In cases of untreated diabetes mellitus, plasma glucose level exceeds the reabsorbing capacity of the proximal tubule, resulting in the presence of unabsorbed glucose in urine. This cause could be rejected as this should be accompanied by the symptoms of initial polyuria which was not the cases in this study. In addition, the symptoms of glucosuria should exist much earlier than the proteinuria which would be found only in the advanced stage of diabetes mellitus. Yet, proteinuria existed before glucosuria in the present study. Another cause of glucosuria is the primary renal impairment in which there is a proximal tubular defect in glucose reabsorption. Plasma glucose is able to pass through the glomerular wall freely by glomerular ultrafiltration. Glucose in the glomerular filtrate is then completely absorbed by the proximal tubule in normal functioning kidneys through active transport. Impairment in the proximal tubule

would result in failure of complete glucose reabsorption (Rose and Rennke, 1994; Walmsley and White, 1983). Nonetheless, the histological findings of subchronic toxicity test revealed no evident lesion in the proximal tubules, except the local cloudy swelling of proximal tubules of several rats in the treatment group fed with Ma-dou-ling extract 10 g/kg. Ma-dou-ling extract might have impaired the glucose reabsorption by impeding the active carrier transport system for glucose without any cell necrosis and atrophy of tubules (Valtin and Schafer, 1995).

Besides glucosuria, proteinuria was noted in the urinalysis of the treatment groups. The normal protein content in urine is extremely low when compared with that in the plasma because proteins of large molecular weight are unable to pass through the glomerular wall whereas those of small molecular weight filtered would then be actively and almost completely reabsorbed by the proximal tubule. Proteinuria can be traced to either glomerular or tubular origins. Proteinuria of glomerular origin is due to an increase in the permeability of the injured glomerular wall to the large proteins which are then excreted. On the other hand, tubular proteinuria relates to the impairment of proximal tubule which results in the failure of reabsorption and excretion of the low molecular weight proteins (Rose and Rennke, 1994). The exact type of the proteinuria caused by Ma-dou-ling was not determined because the Folin-Lowry protein assay performed in the present study was unable to differentiate the two causes (Pesce and First, 1979). In addition, the histological findings did not reveal any lesion in these two parts, except some focal cloudy swelling of the cortical proximal tubules found in a few animals of the 10 g/kg group. Therefore, the exact etiology of proteinuria caused by Ma-dou-ling extract

was not obvious. Nonetheless, the findings of glucosuria might suggest a greater possibility of proteinuria of tubular origin. In addition, the result of acute toxicity also showed damage in renal part of mice treated by Ma-dou-ling - the proximal tubules. Ma-dou-ling extract may probably impaired this particular part, causing the failure of reabsorption of glucose and protein.

Originally, the purpose of measuring the urine volume was to calculate the total amount of urinary protein and glucose. The present study also showed an increasing trend of urine volume in all the treatment groups, depending on dose and duration of the treatment. Significant polyuria was noted since 40 days after the onset of the oral administration of Ma-dou-ling extract. This showed there was an impairment in the concentration ability of the kidney.

From the pathological findings in the acute toxicity test, the impairment of renal functions by Ma-dou-ling extract in the subchronic toxicity test might be primarily due to damage of the proximal tubules. As mentioned before, the proximal tubules are responsible for the active secretion and metabolism of organic ions and harmful compounds. Therefore, the proximal tubules are vulnerable to the high concentration of toxins and their metabolites (Swan and Bennett, 1993; Valtin and Schafer, 1995). It might be the target site of the toxicants and their active metabolites in Ma-dou-ling extract.

In comparison with the renal impairment by AA, there was similarity in the results of the urinalysis. Proteinuria, glucosuria, and impaired urinary concentrating ability were resulted from the subchronic toxicity of AA (Mengs and Stotzem, 1992; Peters and Hedwall, 1963) and Ma-dou-ling extract. However, no evident necrotic lesion was found in the cortical renal tubules of rats fed with Ma-dou-ling extract, whereas the damage prominent in rats intoxicated by 25 mg/kg i.g. but not by 5 mg/kg i.g. AA 1 month after subchronic intragastric administration (Mengs and Stotzem, 1992). Another investigation on subchronic toxicity of AA by Mengs et al. (1982) did not demonstrate any impairment of renal function and lesion in the renal cortex at a lower dosage of 10 mg/kg i.g. This suggests that a higher dosage or longer duration of treatment of Ma-dou-ling extract might be required to result in the same histological findings.

As compared with the Chinese herbal nephropathy (CHN) and Balkan endemic nephropathy (BEN), the clinical results of urinary glucose and protein contents in rats fed with the Ma-dou-ling extract did show an elevated level. Yet, reduced kidney size and interstitial renal fibrosis, the characteristics of CHN and BEN, were not observed in the present subchronic administration of Ma-dou-ling extract on experimental rats.

In addition to impairment in renal functions, subchronic administration of Ma-dou-ling extract induced the development of squamous cell carcinoma in the forestomach. In rats, the stomach consists of two parts: the forestomach (non-glandular portion) and the body (glandular portion). The luminal surface of the forestomach is the keratinized stratified squamous epithelium, whereas the body contains the modified columnar

epithelium (Craddock, 1993). The present study revealed that Ma-dou-ling extract induced papillomas and carcinomas in the forestomach without invasion to the stomach body. This indicated the carcinogenic action of Ma-dou-ling extract was specific to the region of the forestomach.

Hyperkeratosis, hyperplasia, carcinoma and metastasis were the histological findings in the forestomach of the treatment groups. The extent of these pathological changes in the different treatment groups depended on dosage and duration of treatment. Such changes appeared to follow the sequence of histological changes in esophageal and forestomach cancer formation (Craddock, 1993, Mengs, 1983), starting with normal stage, and then advancing to mild hyperplasia with hyperkeratosis, marked hyperplasia with hyperkeratosis, early carcinoma *in situ,* and finally invasive carcinoma. Therefore, after 3-month subchronic intragastric administration of Ma-dou-ling extract, only mild hyperplasia with hyperkeratosis were found in the small dosage group (2 g/kg i.g.) whereas marked hyperplasia and invasive carcinoma were noted in the medium (4 g/kg i.g.) and higher (10 g/kg i.g.) dosage groups. It would be plausible to envisage the development of carcinoma in the 2 /kg group if the present study had been continued for a longer period of time.

The findings of carcinomas formation in the treatment groups with dosage as low as 4 g/kg i.g. after treatment for 3 months indicated that the Ma-dou-ling extract contained a potent carcinogenic substance. Aristolochic acid (AA) in the Ma-dou-ling extract was probably, one if not the only, cause for the forestomach carcinomas. Findings

of the investigation by Mengs (1983) and Mengs et al. (1982) on the carcinogenic action and histopathogenesis of rat forestomach carcinoma induced by AA bear much similarities to the macroscopic and microscopic changes in rats fed with the Ma-dou-ling extract.

Measurement of the serum AST and ALT activities was used in the present study to investigate the liver lesion. As mentioned in Section 1.4.2.3, elevated serum enzyme activities indicated an increase in the corresponding serum enzyme level. This in turn indicated there was damage of the organ to which the enzyme is specific. In the present study, the serum ALT activities in the 10 g/kg group was elevated after 1.5 and 3 months of subchronic treatment with the Ma-dou-ling extract, while no increase was noted in the serum AST activities. The raised serum ALT activities indicated an increase in the amounts of ALT level in the bloodstream. As ALT is specific to the liver, this result showed evidence of liver damage in the 10 g/kg group. On the other hand, there was no significant elevation in the serum AST activities, which showed no indication of increasing serum AST level. The two transaminase, ALT and AST, have different intracellular localization, with ALT present only in cytosol while AST in both cytosol and mitochondria. With mild hepatocelluar damage, ALT levels are higher than AST while the elevation of the latter was found in more severe cellular necrosis due to the release of mitochondrial enzymes (Walmsley and White, 1983; Zilva and Panail, 1975). Thus, there was mild hepatocellular damage in rats of the 10 g/kg group after 1.5- and 3-month subchronic treatment with the Ma-dou-ling extract.

Histological study further provided information about the degree of hepatic lesion. No hepatocellular necrosis was found in the microscopic examination in all the treatment groups. The absence of hepatocellular necrosis could well explain the sole elevation in ALT level but not in AST level. The hepatic damage by the Ma-dou-ling extract was not severe to the extent of cell necrosis to cause a release of mitochondrial AST. Therefore no change in serum AST level was noted. For the elevation of serum ALT, this could be accounted for by their leakage from hepatocytes. Subjected to toxin, membrane permeability of the hepatocytes will be increased and thus the cytosol ALT leaks out to the serum while the hepatocytes are intact. (Hørder and Wilkinson, 1979; Jones and Berk, 1979).

Dark brown urine color found in the groups fed with Ma-dou-ling extract 4 and 10 g/kg also provided an indication of hepatocellular damage. In urine of normal subjects, little urobilinogen is excreted by the kidney. The urobilinogen is oxidized to urobilin which is a brown pigment. For those having haemolysis or hepatocellular diseases, an increased urobilinogen content will be found in their urine, which will then become deep orange-brown (Walmsley and White, 1983). The deep orange-brown urinary color in the groups fed with Ma-dou-ling extract 4 and 10 g/kg in this experiment might be due to the hepatocellular damage when taking the result of serum AST assay into consideration. As mentioned before, haemolysis can be indicated by the elevated serum AST lever. Thus, the absence of raised serum AST level excluded the cause of haemolysis.

From the results of the clinical biochemical test (AST and ALT), histological findings and observation of urine color, it could be concluded that the liver of the 10 g/kg group was damaged to an extent of leaking ALT into the bloodstream and causing the impairment of re-excretion of the urobilinogen without any severe hepatocellular necrosis.

In addition to liver, AST is also found in skeletal and cardiac muscles, pancreas as well as erythrocytes. Thus, absence of elevation of serum AST level also suggested no damage to these tissues.

4.3.3 Reproductive toxicity

Ma-dou-ling extract showed a dose-dependent anti-fertility effect on mice. It reduced the number of pregnant mice at a dosage as low as 4.15 g/kg i.g. and produced a 100 % anti-fertility effect at 19.2 g/kg i.g. and above. This indicated that an active constituent was present in Ma-dou-ling extract. Yet, lethality of mice were observed at 32 g/kg i.g.

Ma-dou-ling extract did not reduce the number of implantation sites in pregnant mice. In addition, the extract also did not cause any malformation of fetus.

In the traditional Chinese medical literature, it was stated that Ma-dou-ling had to be used with caution for pregnant women. In the present study, Ma-dou-ling extract

indeed exerted anti-fertility effect at a relatively low dosage (4.15 g/kg i.g.) and could reach 100% activity at 19.2 g/kg i.g. The study confirmed the caution made in traditional Chinese medicine.

Chapter Five: Conclusion

From the results of acute, subchronic and reproductive toxicity studies, the Ma-dou-ling extract showed a higher toxicity than the Guan-mu-tong extract.

In acute toxicity test, even the highest possible dosage of Guan-mu-tong extract did not result in lethality of all the mice dosed (337.72 g/kg i.g.). The lethal dose thus could not be clearly defined. The 3-month subchronic toxicity test of Guan-mu-tong extract did not show any toxic effects on the female rats. For rats treated with Guan-mu-tong extract, there was no reduction in body weight gain, no elevated urinary volume, glucose content, and no raised serum AST and ALT activities except some fluctuations in the urinary protein content. In addition, gross findings at autopsies did not reveal any lesion in major organs. Histopathological study on the liver and kidney did not show any damages by Guan-mu-tong extract. These findings showed that Guan-mu-tong extract had no or only a very mild renal toxic effects on female rats. In the reproductive toxicity test, Guan-mu-tong extract exerted an anti-fertility effect on mice at fairly high dosages, at which level mortality was also resulted.

On the contrary, a single dose of Ma-dou-ling extract (51.2-100 g/kg i.g.) led to mortality of mice in the acute toxicity test. The LD_{50} with confidence limit of 95 % was 64.74±1.22 g/kg i.g. Animals died mainly on the third to sixth day after dosing. In addition, there was a reduction in body weight of the moribund and surviving animals. Gross and histopathological examination revealed that the kidney was the target organ of

the toxicants. The clinical observation, body weight together with the gross and histopathological findings showed that the animals probably died due to acute renal failure. This may indicate that a single dose of the Ma-dou-ling extract caused lethality in animals by impairing the renal functions. The subchronic test of the Ma-dou-ling extract showed toxic effects on the female rats even at the lowest dosage in this study. There was a reduction in body weight gain. Elevated urinary volume, glucose and protein indicated impairment of renal function by the Ma-dou-ling extract. Raised serum ALT activities also reflected the damage caused by the Ma-dou-ling extract to liver tissues. Besides, papilloma and carcinoma in stomach showed that the Ma-dou-ling extract contained carcinogens. The findings from the subchronic study proved that Ma-dou-ling is a potent Chinese herbal drug and would be harmful if consumed for prolonged period of time. For the reproductive toxicity test, the Ma-dou-ling extract showed a dose-dependent reduction in fertility rate at relatively lower dosages. The result showed that Ma-dou-ling should be prescribed in caution for pregnant women.

In comparison with AA, the Ma-dou-ling extract showed a similar pattern of toxic effects in acute, subchronic and reproductive toxicity tests. This implies that AA would be one of main active constituents in Ma-dou-ling contributing the toxic effects in the toxicity studies. This was further supported by the quantitative findings from HPLC analysis which showed the Ma-dou-ling extract to have a higher AA content than Guan-mu-tong extract and thus the toxicity tests of the latter showed a relative lower harmful effects. However, it should be emphasized that the result only revealed the toxicity of

the aqueous extract of the two herbal drugs but not the crude drug as the result of HPLC showed that crude drug Guan-mu-tong had a relatively higher AA content.

In the 3-month subchronic test of the Ma-dou-ling and Guan-mu-tong extracts on rats did not resulted in renal interstitial fibrosis which is the characteristic finding in both BEN and CHN. A chronic toxicity test, 9- or 12-month period, might be required to investigate the long-term effect of the two Chinese herbs of *Aristolochia* species.

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Appendix

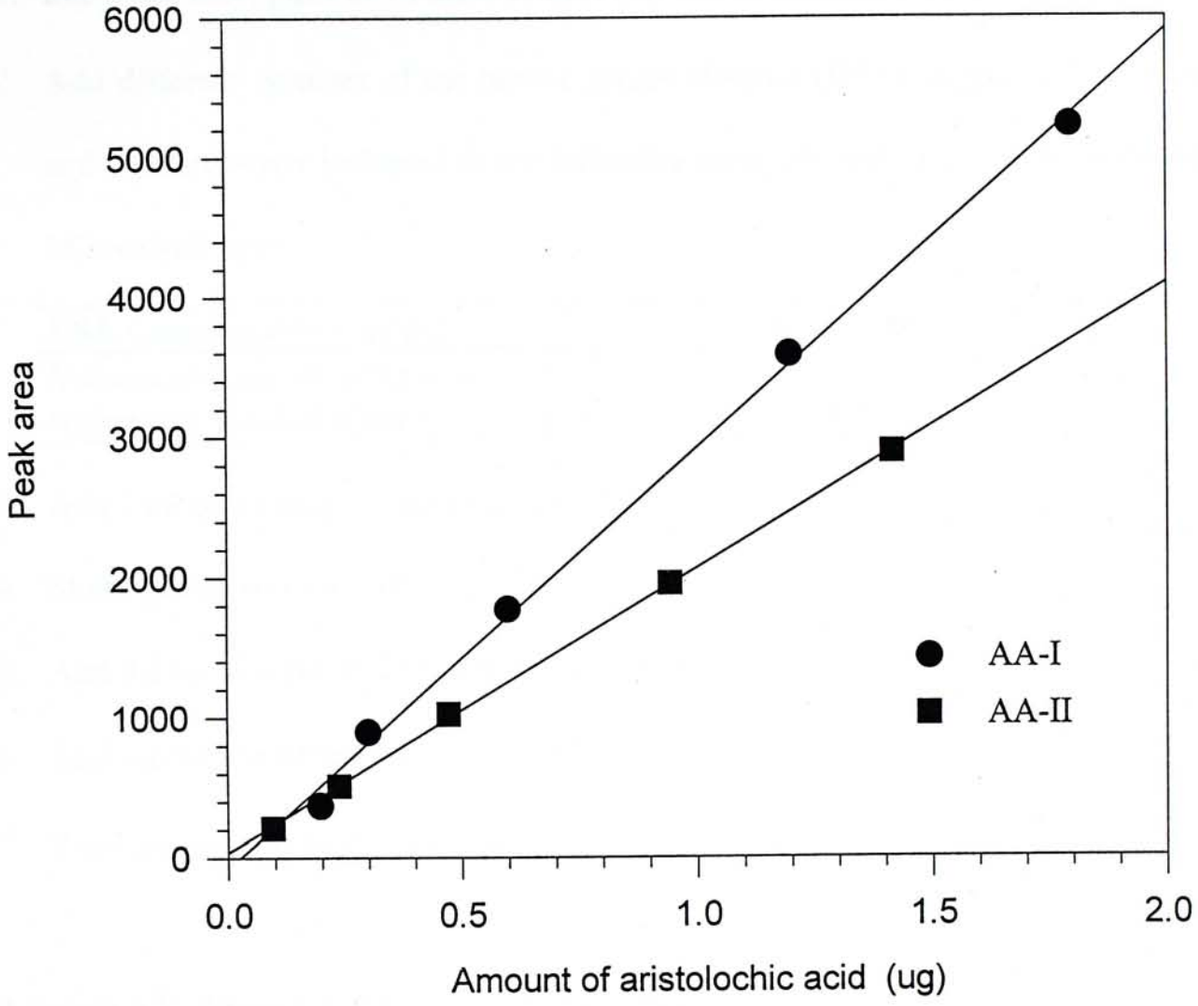

Figure A1: Calibration curves of aristolochic acid I (AA-I) and II (AA-II) using profile of HPLC and UV 254 nm.

Appendix A: Procedure on determining the total urinary protein.

1. Into a test tube, pipet 20 μl urine sample and 180 μl distilled water.
2. Add different volumes of the bovine serum albumin (BSA) (Sigma, USA) solution and distilled water indicated in the following table into test tubes for the preparation of standard curve:

BSA Concentration (mg/ml)	0	0.2	0.4	0.6	0.8	1
Volume of 1 mg/ml of BSA (μl)	0	40	80	120	160	200
Volume of distilled water (μl)	200	160	120	80	40	0

3. Add 2 ml of solution I* into each test tube.
4. Shake gently and wait for 10 minutes.
5. Add 0.2 ml of solution II** into each test tube.
6. Shake gently and then leave in a 37°C water bath for 30 minutes.
7. Read and record absorbance at 650 nm.

* Solution I: Reagent A:B:C = 1:1:100 (freshly prepared) where

Reagent A: 1% cupric sulfate ($CuSO_4.5H_2O$) (Sigma, USA)

Reagent B: 2% sodium potassium tartrate ($C_4H_4O_6KNa.4H_2O$) (Sigma, USA)

Reagent C: 2% sodium carbonate ($Na_2CO_3.10H_2O$) (Peking Analytical Reagent, China) in 0.1 M sodium hydroxide(BDH, England)

**Solution II: Folin-Ciocalters phenol reagent (dilute one time with distilled water before use) (BDH, England)

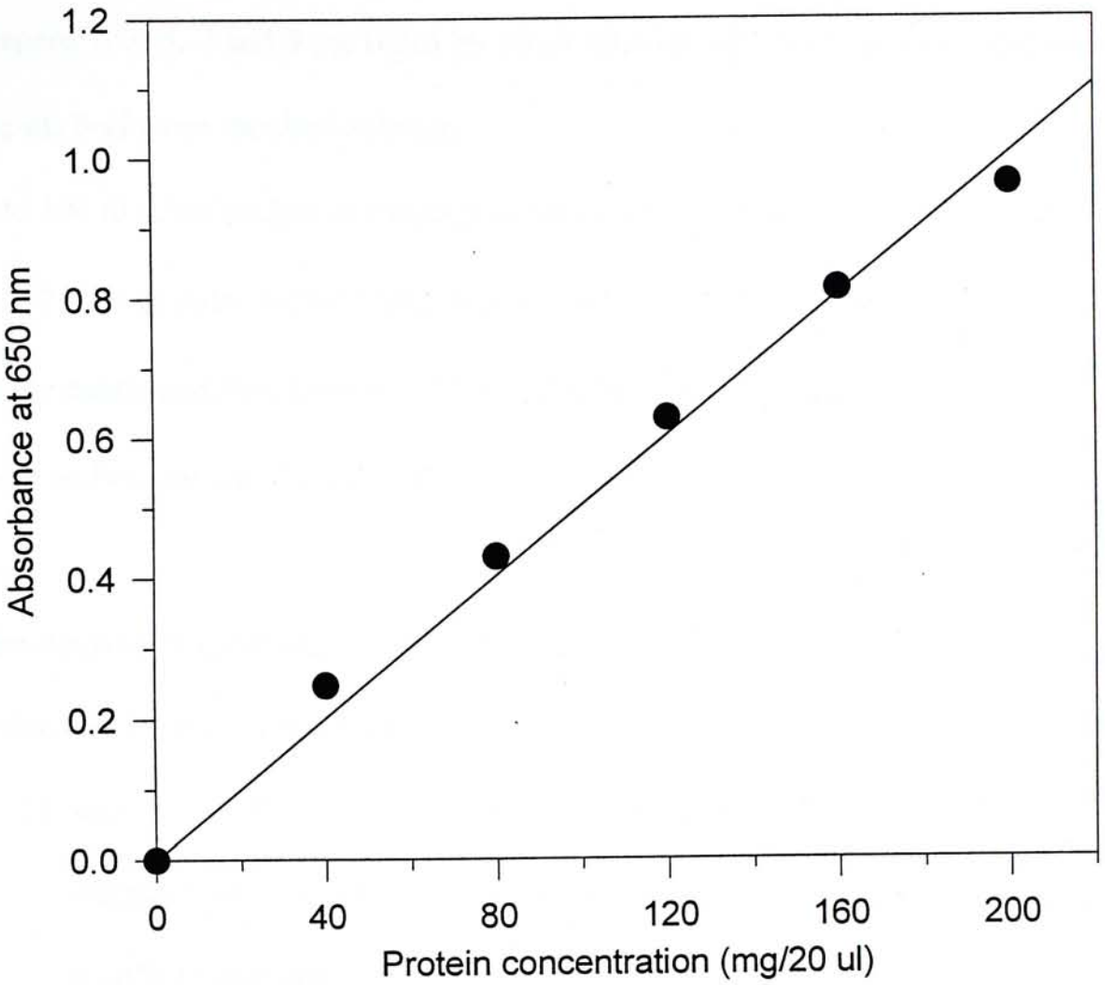

Figure A2: Calibration curve of total urinary protein.

Appendix B: Procedure on determining the total urinary glucose using Sigma diagnostic kits.

1. Prepare 1, 3, 5, 7 and 9 μg/100μl by serial dilution with 0.1% benzoic acid from 100 mg/dL β-glucose standard solution.
2. Add 100 μl urine sample or standard solution into a test tube.
3. Add 2.5 ml of color-enzyme reagent solution* into each test tube.
4. Shake gently and then leave in a 37°C water bath for 30 minutes.
5. Read and record absorbance at 450 nm.

* Color-enzyme reagent solution was then prepared by combining 100 ml of enzyme solution and 1.6 ml of color regent solution where

    1) enzyme solution was prepared by dissolving contents of 1 capsule of enzymes which contained peroxidase and glucose oxidase to 100 ml distilled water and
    2) color reagent solution was prepared by reconstituting one vial of o-Dianisidine Dihydrochloride with 20 ml distilled water. Aliquots of 1.6 ml were kept in eppendorf tube storing at 4°C.

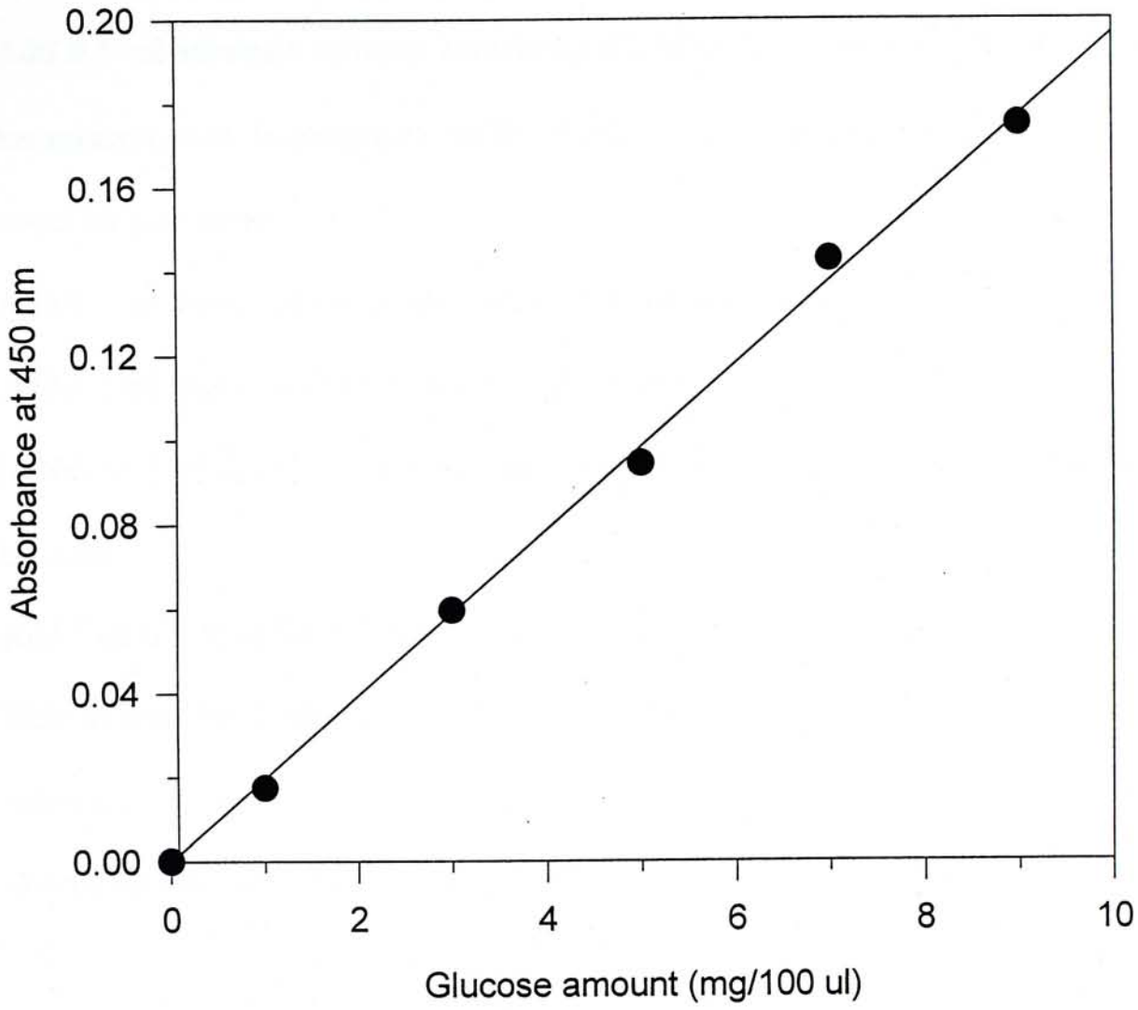

Figure A3: Calibration curve of total urinary glucose.

Appendix C: Procedure on determining the activity of aspartate aminotransferase (AST).

1. Add 0.5 ml substrate solution containing 0.2 M of DL-aspartate and 1.8 mM of α-ketoglutaric acid, in phosphate buffer of pH 7.5, into a test tube and place in a 37°C water bath to warm.
2. Add 0.1 ml serum. Shake gently and leave in the water bath.
3. Add 0.5 ml Sigma Color Reagent which contains 1 mM of 2,4-dinitrophenlhydrazine (DNP) in 1 M hydrochloric acid. Shake gently and leave at room temperature for 20 minutes.
4. Add 5 ml 0.4 M sodium hydroxide. Mix by inversion.
5. Wait at least for 5 minutes. Read and record absorbance at 505 nm using water as reference.
6. Determine AST activity from the calibration curve (Figure A4 and A5).

Appendix D: Procedure on determining the activity of alanine aminotransferase (ALT).

1. Add 0.5 ml substrate solution containing 0.2 M of DL-alanine and 1.8 mM of α-ketoglutaric acid, in phosphate buffer of pH 7.5, into a test tube and place in a 37°C water bath to warm.
2. Add 0.1 ml serum. Shake gently and leave in water bath.
3. Add 0.5 ml Sigma Color Reagent which contains 1 mM of 2,4-dinitrophenlhydrazine (DNP) in 1 M hydrochloric acid. Shake gently and leave at room temperature for 20 minutes.
4. Add 5 ml 0.4 M sodium hydroxide. Mix by inversion.
5. Wait at least for 5 minutes. Read and record absorbance at 505 nm using water as reference.
6. Determine ALT activity from the calibration curve (Figure A4 and A5).

Appendix E: Procedure for preparing a calibration curve for the measurement of aspartate aminotransferase (AST) & alanine aminotransferase (ALT) activities.

1. Add different volumes of the solutions indicated in the following table into test tubes:

Tube no.	*Calibration standard solution (ml)	**Substrate (ml)	Distilled water (ml)	Serum AST activity (SF Units/ml)	Serum ALT activity (SF Units/ml)
1	0	0.5	0.1	0	0
2	0.05	0.45	0.1	20	23
3	0.1	0.4	0.1	55	50
4	0.15	0.35	0.1	95	83
5	0.2	0.3	0.1	148	125
6	0.25	0.25	0.1	216	-

* Calibration standard solution is 1.5 mM of sodium pyruvate , in phosphate buffer of pH 7.5.

** Substrate solution containing 0.2 M of DL-aspartate and 1.8 mM of α-ketoglutaric acid for AST and substrate solution containing 0.2 M of DL-alanine and 1.8 mM of α-ketoglutaric acid for ALT.

2. Add 0.5 ml color reagent to each tube. Shake gently and leave at room temperature for 20 minutes.
3. Add 5 ml 0.4 M sodium hydroxide solution to each tube and mix by inversion.
4. Wait at least 5 minutes.
5. Read and record the absorbance at wavelength 505 nm using water as reference.
6. Plot AST and ALT calibration curves of absorbance values versus the corresponding units of AST and ALT respectively (Figure A4 and A5).

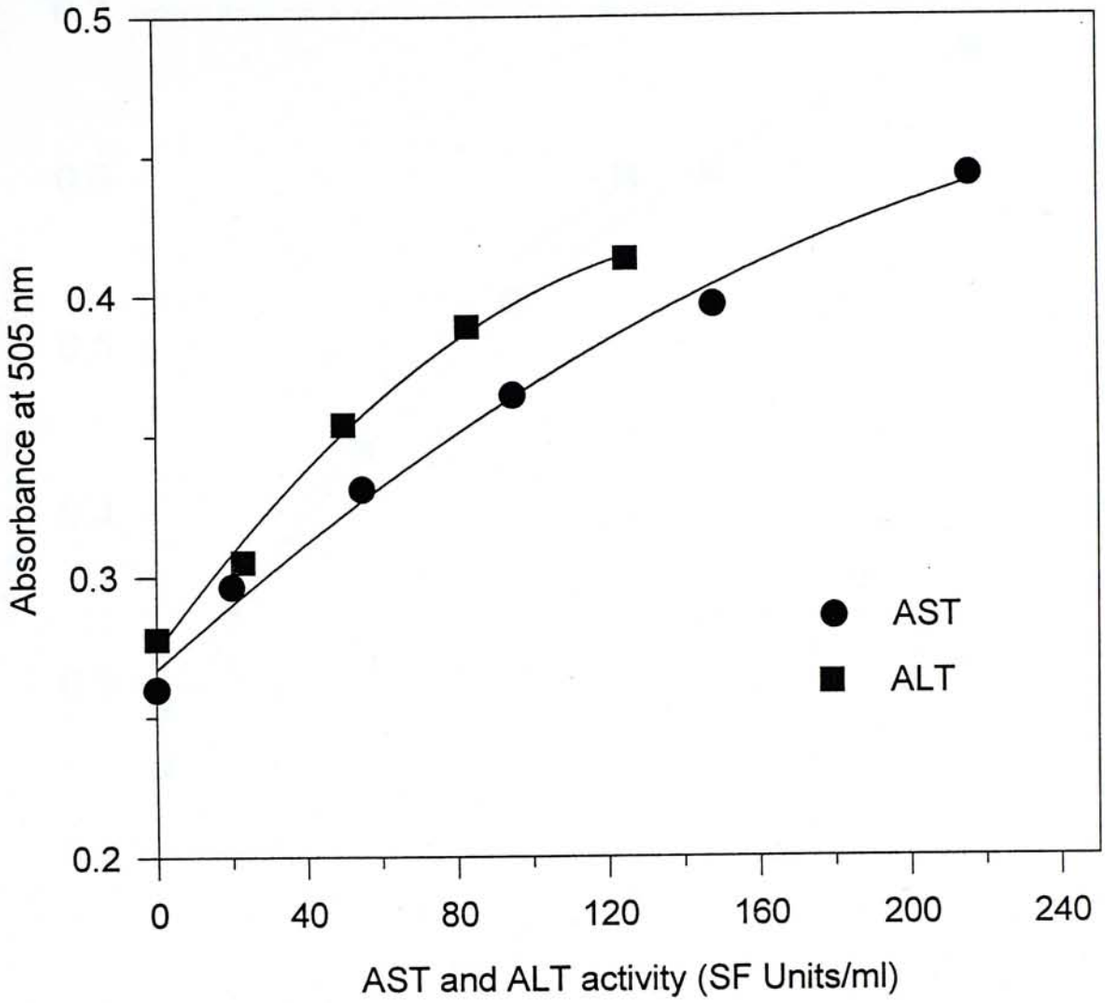

Figure A4: Calibration curves of aspartic aminotransferase (AST) and alanine aminotransferase (ALT) activites for subchronic toxicity test of Guan-mu-tong extract.

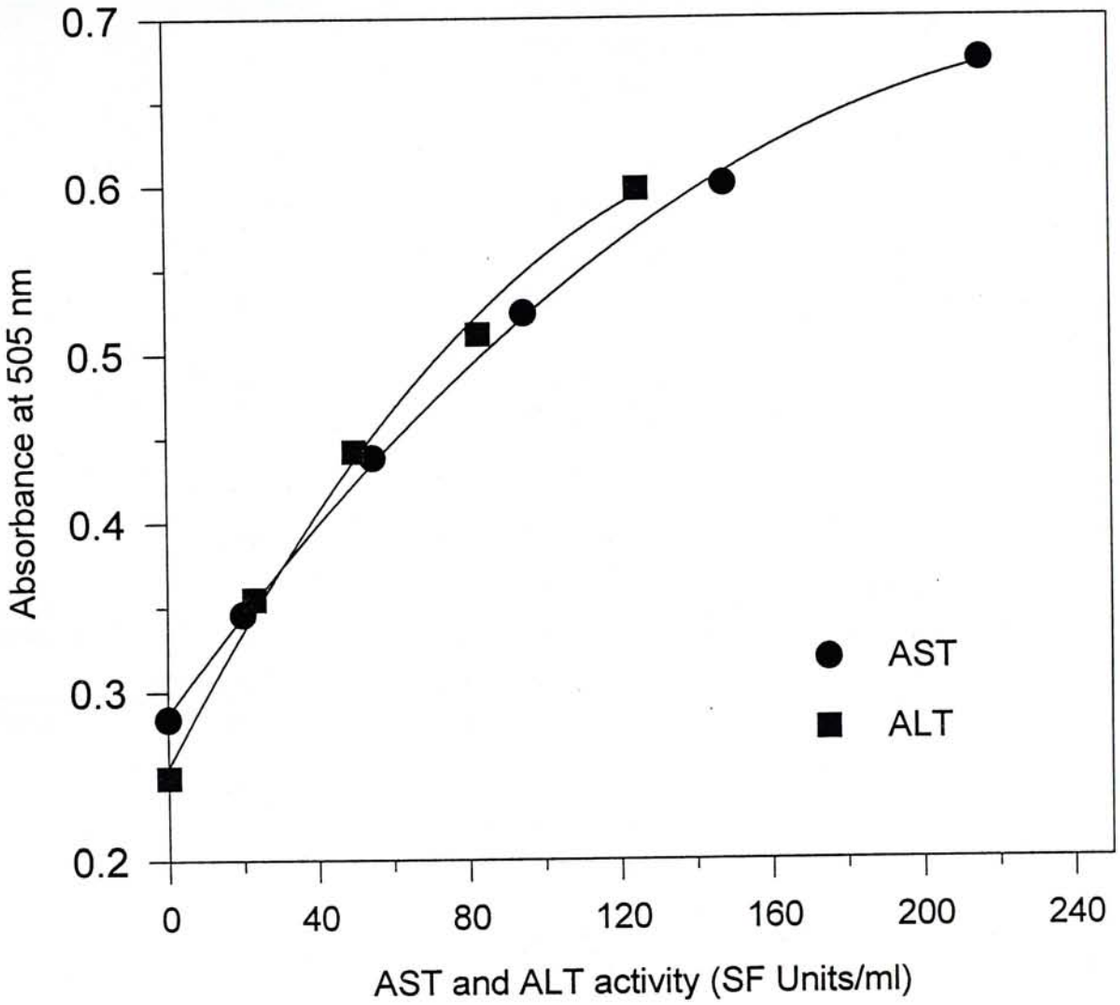

Figure A5: Calibration curves of aspartic aminotransferase (AST) and alanine aminotransferase (ALT) activities for subchronic toxicity test of Madou-ling extract.

Appendix F: Procedure on tissue preparation for light microscopic study.

Procedure	Reagent		Time
Fixing	10 % buffered formalin		>24 hours
Processing	Dehydration	50 % Ethanol	2 hr.
		70 % Ethanol	2 hr.
		85 % Ethanol	2 hr.
		95 % Ethanol	1 hr.
		100 % Ethanol	1 hr.
		100 % Ethanol	1 hr.
	Clearing	Xylene:Ethanol (1:1)	30 min.
		Xylene	30 min.
		Xylene	30 min.
	Infiltration	Paraffin wax	1 hr.
		Paraffin wax	1 hr.
		Paraffin wax (Vacuum aspiration)	30 min.
	Embedding	Paraffin wax (allow to cool and trim)	-
Sectioning	Section at 5 μm thick, adhere on slides and dry on warm plate overnight.		
Staining	Dewax	Xylene	5 min.
		Xylene	5 min.
	Hydration	100 % Ethanol	1 min.
		95 % Ethanol	1 min.
		70 % Ethanol	1 min.
		50 % Ethanol	1 min.
		30 % Ethanol	1 min.
		running tap water	1 min.
	Staining	Mayer's Hematoxylin	10 min.
		running tap water	1 min.
		1 % acid alcohol	~5 sec.
		running tap water	1 min.
		Scott's tap water	1 min.
		running tap water	1 min.
		0.5 % aqueous eosin	4 min.
		running tap water	1 min.
	Dehydration	70 % Ethanol	~5 sec.
		95 % Ethanol	~5 sec.
		100 % Ethanol	1 min.
		100 % Ethanol	2 min.
		Xylene:Ethanol (1:1)	2 min.
		Xylene	2 min.
		Xylene	2 min.
Mounting		Canada Balsam	-